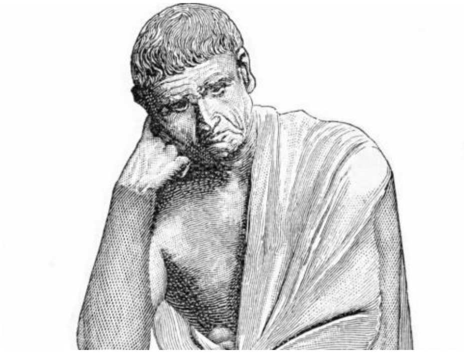
From single molecules to heart disease

Fundación
Centro Nacional de
Investigaciones
Cardiovasculares
Carlos III

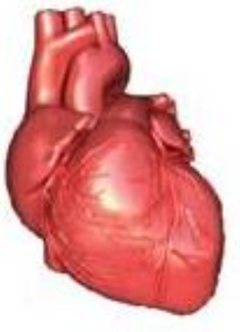
Jorge Alegre-Cebollada

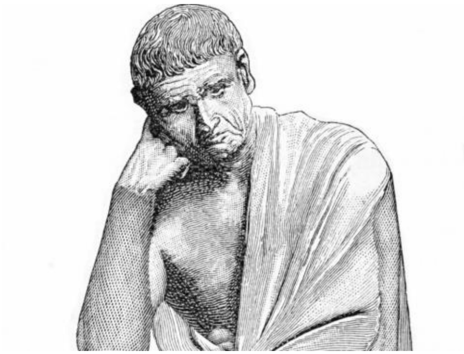
A thought experiment involving time travel



How long would it take for them to understand how a Ferrari works?

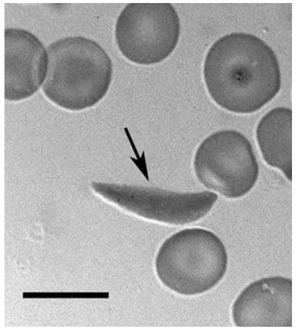
How far are we from understanding the heart?

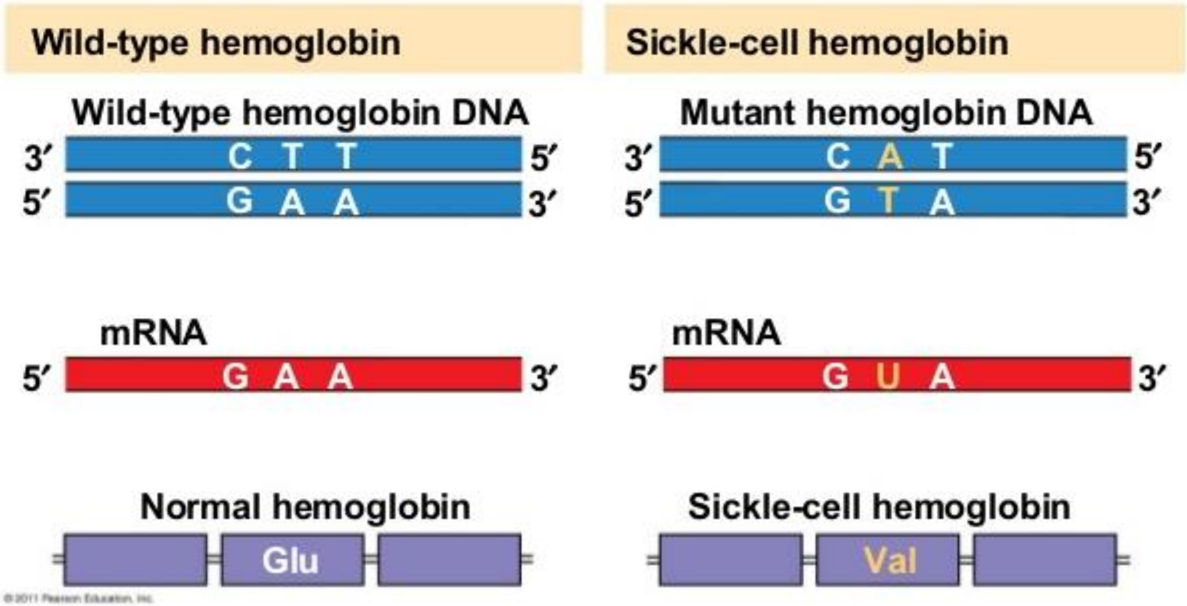




A single amino acid substitution can cause cardiovascular disease

Sickle-cell anemia

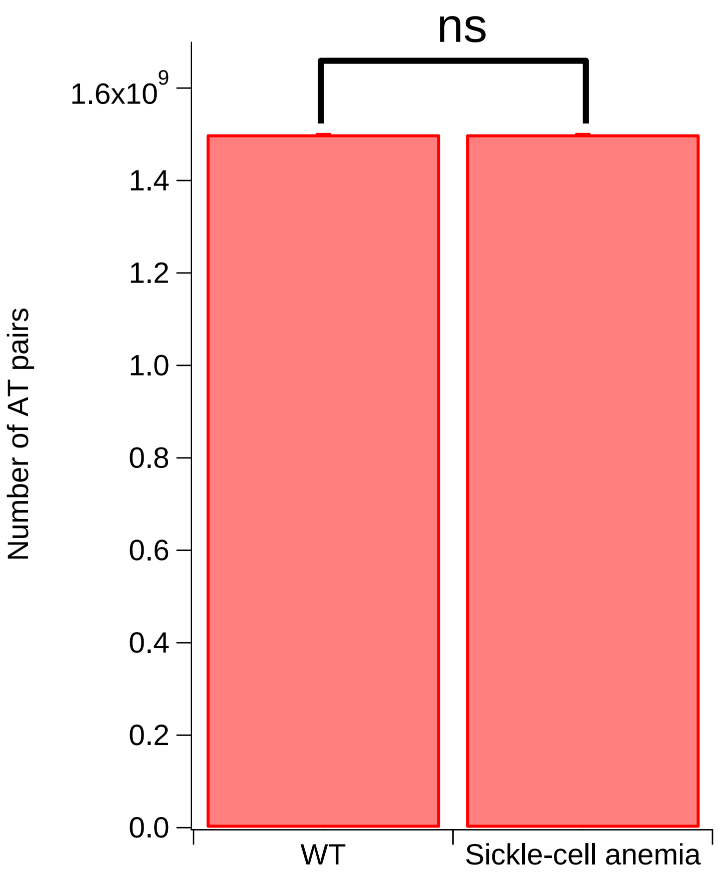




And channelopathies, cardiomyopathies, hypercholesterolemia...

A SINGLE AMINO ACID SUBSTITUTION CAN CAUSE CARDIOVASCULAR DISEASE!!!!

Number of base pairs in the genome: 3×10^9



Number of people in the world: 7×10^9

Genetics *vs.* risk factors

Risk factors

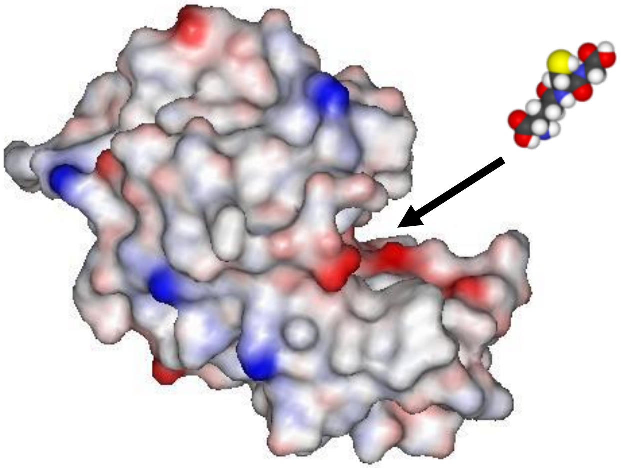
Sudden death

Genotype to phenotype: why do we want to know?

Diagnostic reasons

Polymorphism or pathogenic mutation?

Therapeutic reasons



Drugs that restore healthy phenotype

Why a particular missense mutation causes disease?

1. Decreased thermodynamical stability/protein levels (haploinsufficiency)

   It is considered to be a major driver of pathogenesis JMB 353, 459 (2005).

2. Defective activity (catalytic activity of an enzyme)

   Difficult to predict, challenging to determine experimentally

3. New toxic properties (poison peptide)

   e.g. Hemoglobin

4. Pre-protein effects

   RNA levels, alternative splicing leading to truncations, etc.

5. Defective mechanical properties

   Highly relevant for proteins or the contractile machinery of the sarcomere

Why single molecules?

Maritime routes between New York and San Francisco

Average vs single trajectories

By averaging we loose information

Example listened to Steve Block, a pioneer in single-molecule optical tweezers

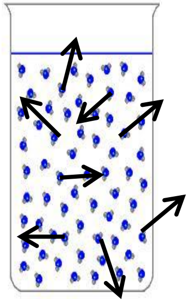
Advantages of single-molecule approaches

1. Novel information that cannot be obtained in bulk

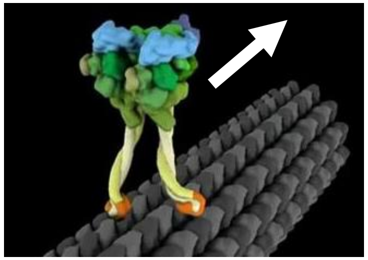
- Access to individual properties that are not accesible to bulk experiments

- Synchronization

- **<u>Access to vectorial properties: movement, force, etc...</u>**



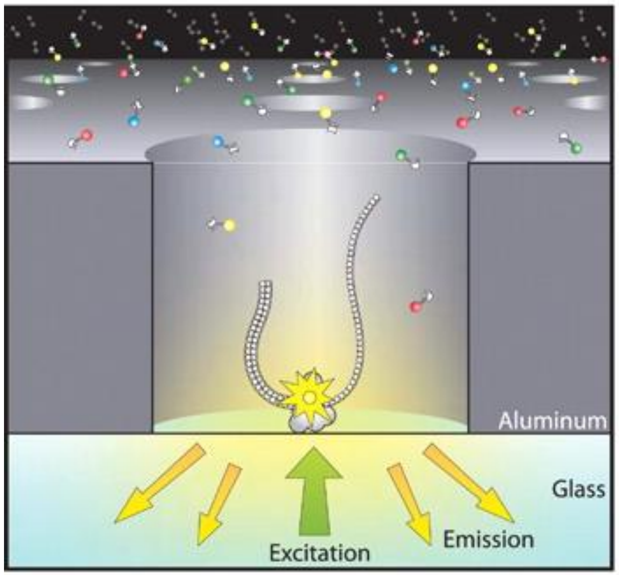
Random orientation of molecules in bulk



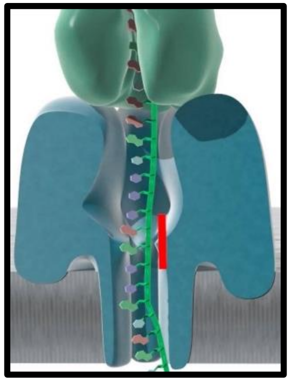
Molecular Motors

Advantages of single-molecule approaches

2. Less material is needed/parallelization: next-generation DNA sequencing



Single-molecule fluorescence

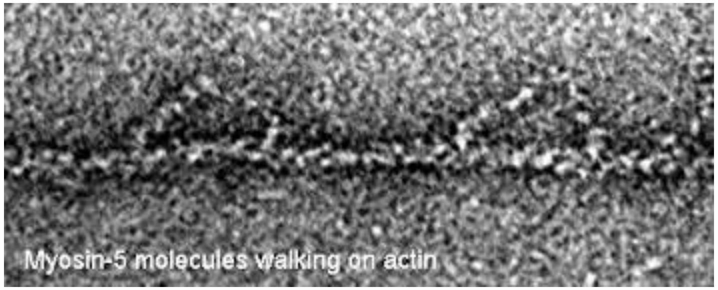


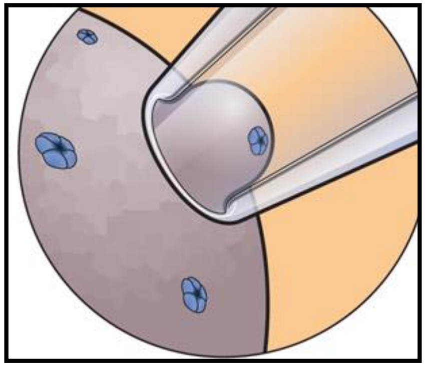
Nanopore technology
(Oxford Nanopores)

Main single-molecule techniques: classification

- Manipulation: application of mechanical forces
  - Atomic Force Microscopy (AFM), magnetic tweezers, optical tweezers, nanopores
- Observation
  - fluorescence

First single-molecule techniques





Electron Microscope
E. Ruska
Nobel Prize in Physics, 1986

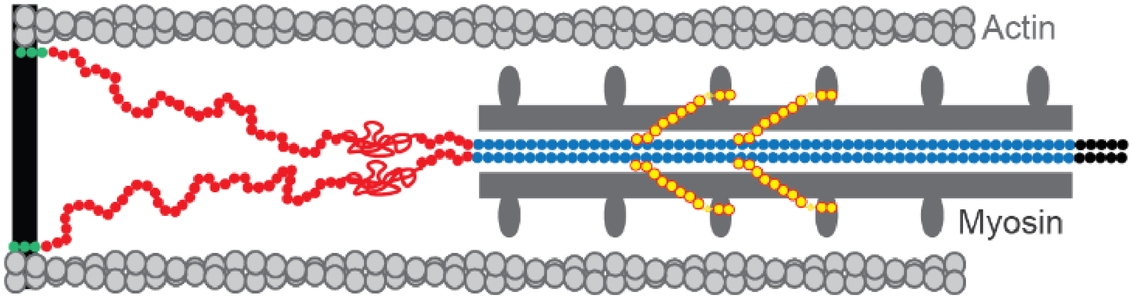
Single-channel patch clamp
E. Neher y B. Sakmann
Nobel Prize in Physiology or Medicine, 1991

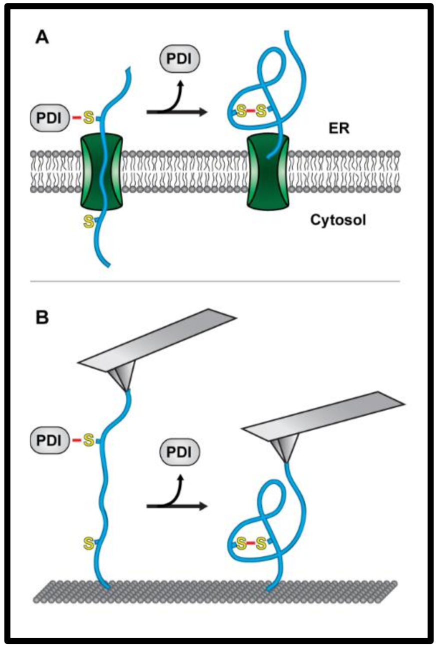
Challenges associated with single-molecule experiments

- Complex instruments and experiments
- Signal is small: how can we tell apart signal from noise?
- New mindset
- New methods of analysis and interpretation of results

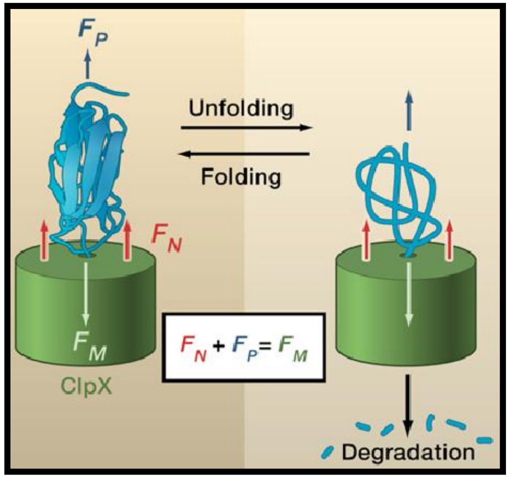
Mechanical forces and proteins: from the cradle to the grave

Muscle activity





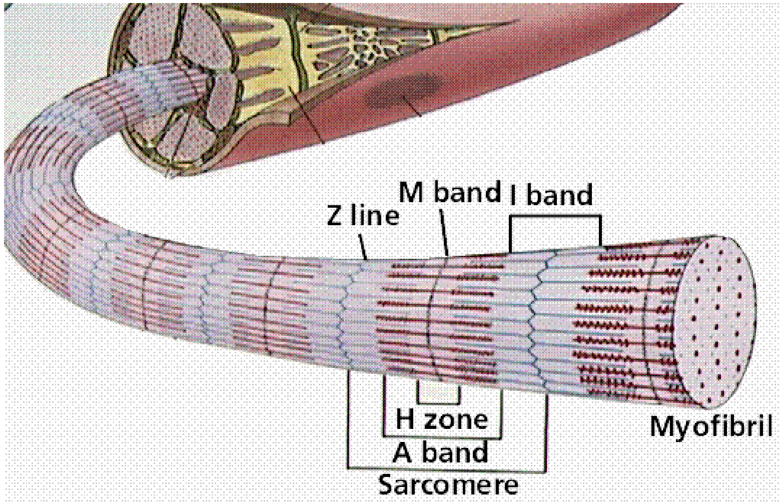
Oxidative folding

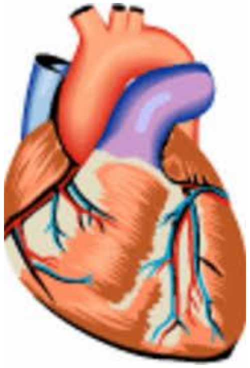


Proteasomal degradation

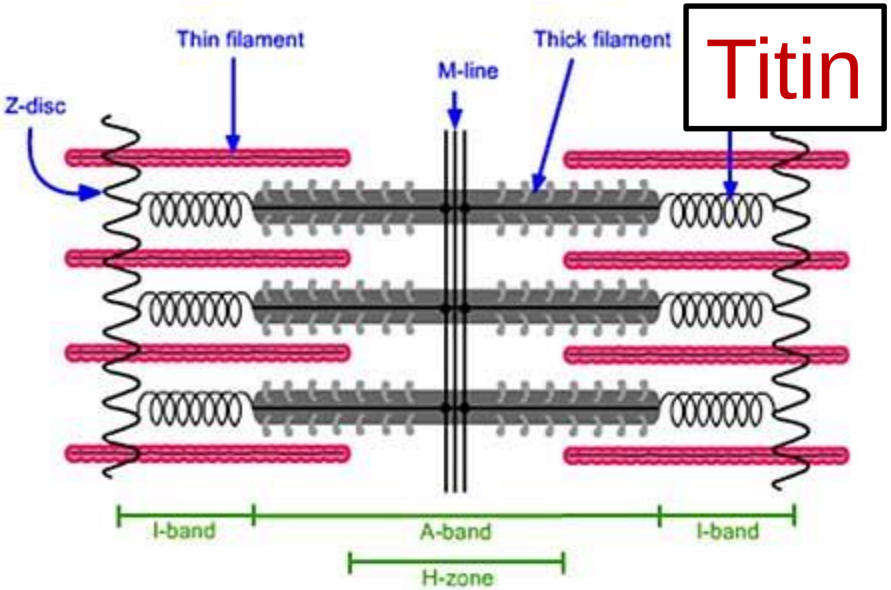
The sarcomere is the functional unit of striated muscle

Skeletal muscle



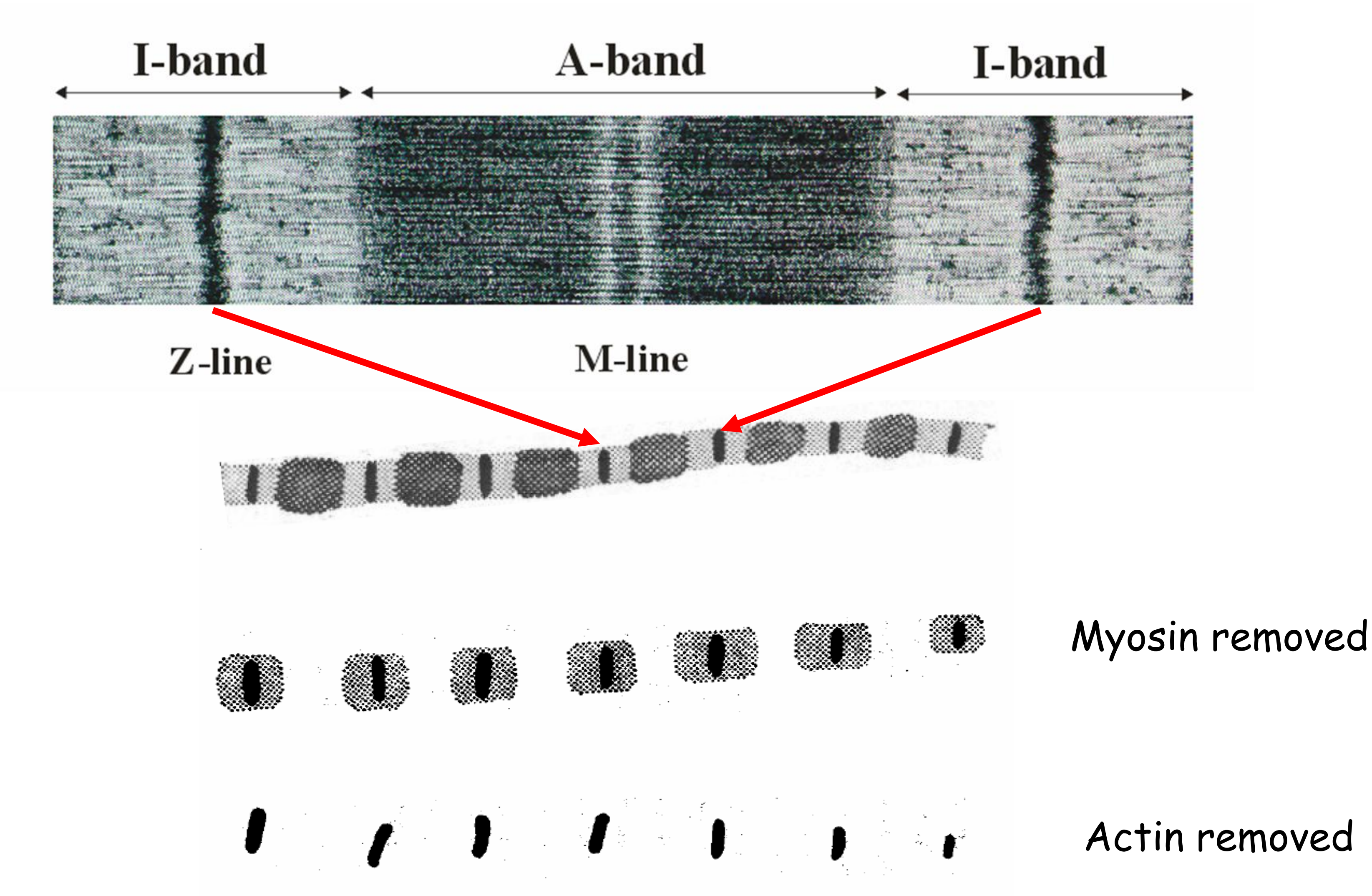


Cardiac muscle

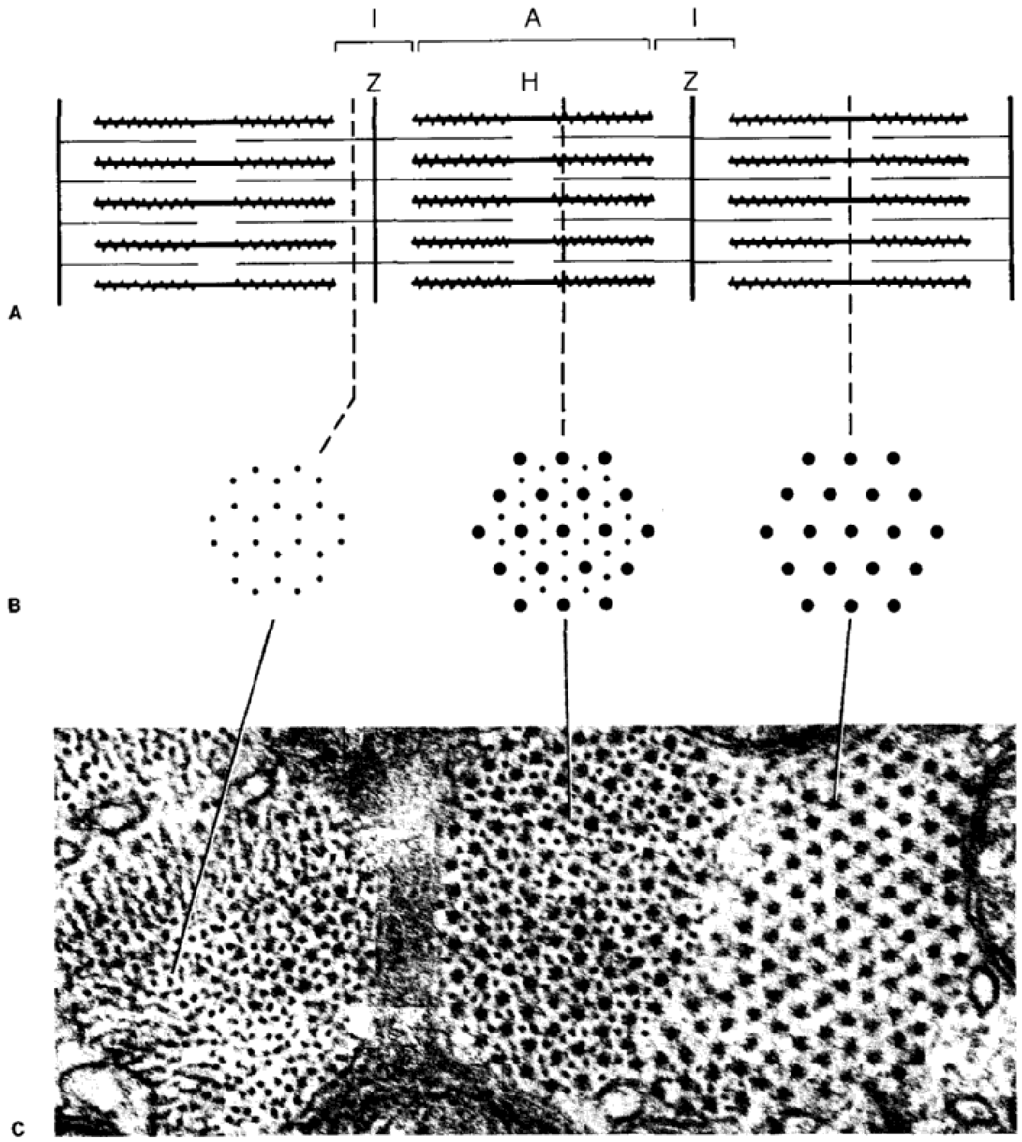


Sarcomere

Sarcomere organization as observed by electron microscopy



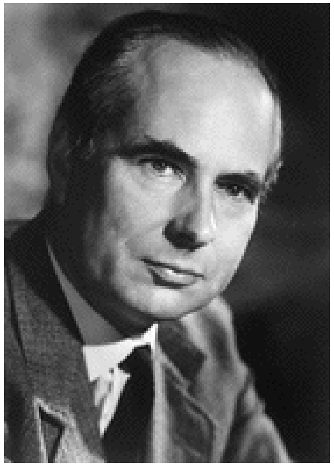
Sarcomere organization as observed by electron microscopy



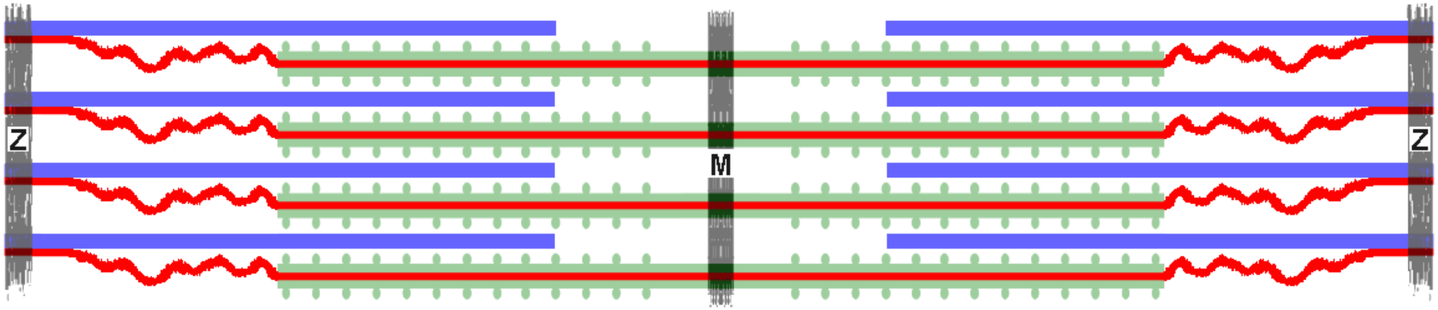
9-3 Organization of the myofibril. (A) Diagram of three sarcomeres, showing thick and thin myofilaments forming I, A, and H bands and Z lines. (B) Imaginary sections through the sarcomere at different levels show profiles of thin (left) and thick (right) filaments, and both types (center). (C) Electron micrograph of a cross section in which the sarcomeres of adjacent myofibrils are out of register and can thus be matched with the corresponding profiles shown above. Spider monkey extraocular muscle. Magnification 100,000×. [Courtesy of L. D. Peachey.]

The sliding filament hypothesis of muscle contraction

Andrew Fielding Huxley



Hugh Huxley



Both authors independently proposed the sliding filament hypothesis in 1952

Testing the sliding filament hypothesis

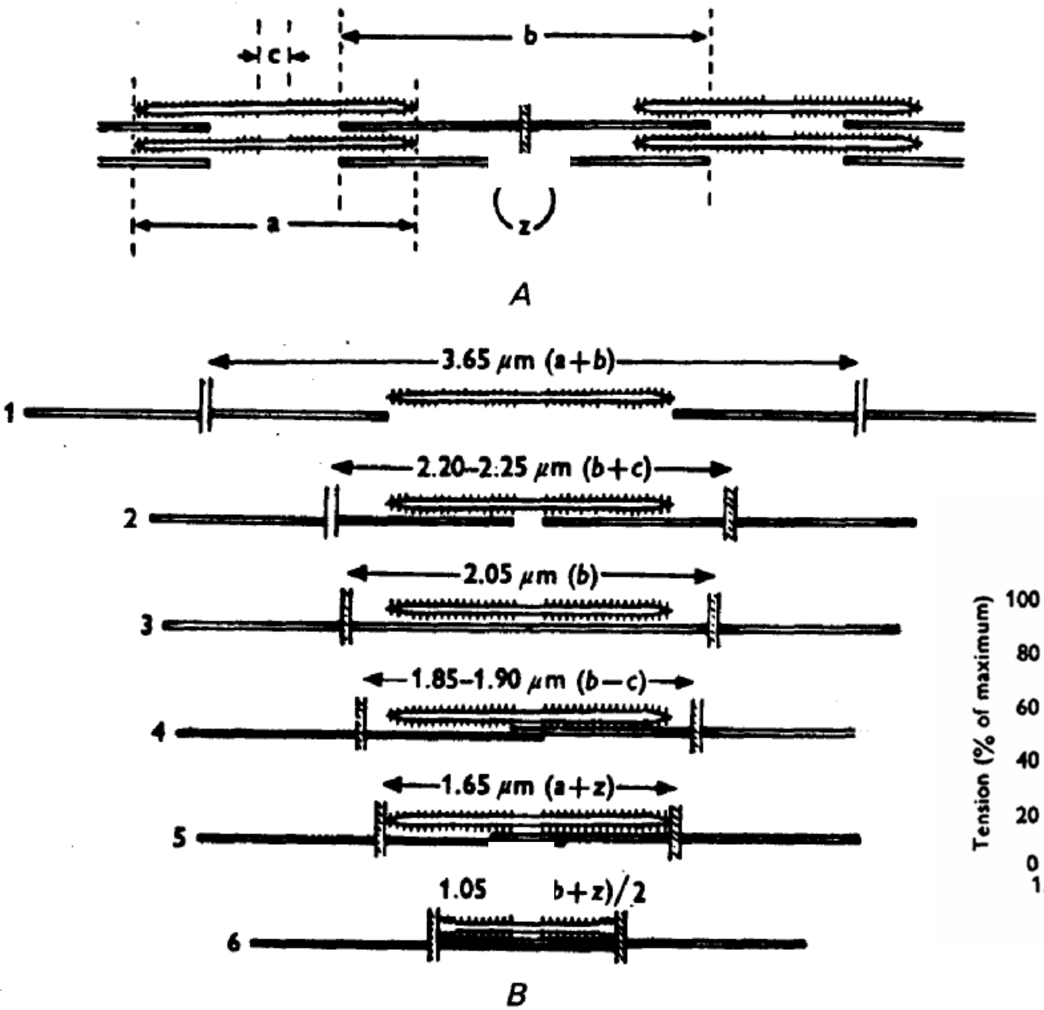
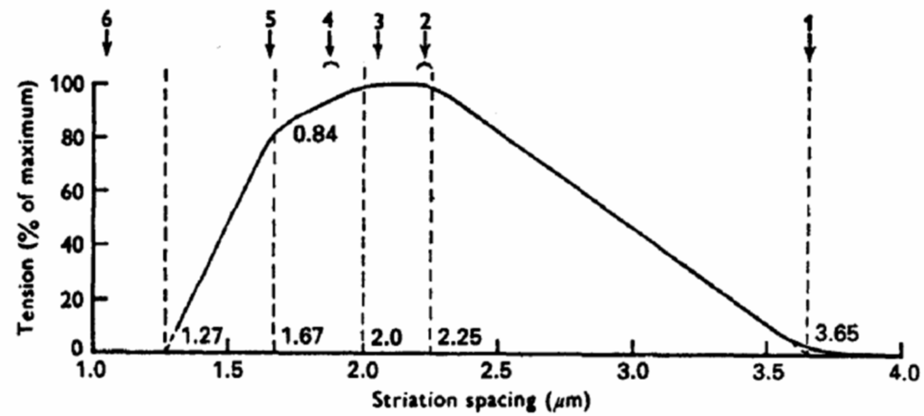
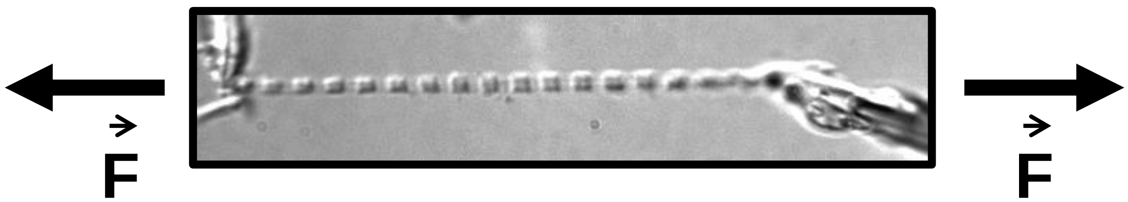


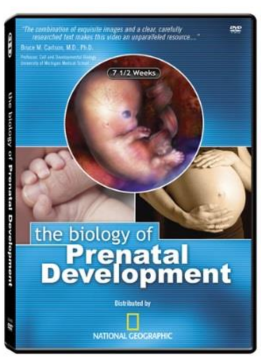
Figure 19.17. Myofilament dimensions in frog muscle. The lower diagram *B* shows the myofilament arrangements at different lengths; the letters *a*, *b*, *c* and *z* refer to the dimensions given in the upper diagram *A*. The sarcomere lengths corresponding to the positions labelled 1 to 6 are indicated by the arrows in fig. 19.16. (From Gordon *et al.*, 1966*b*.)

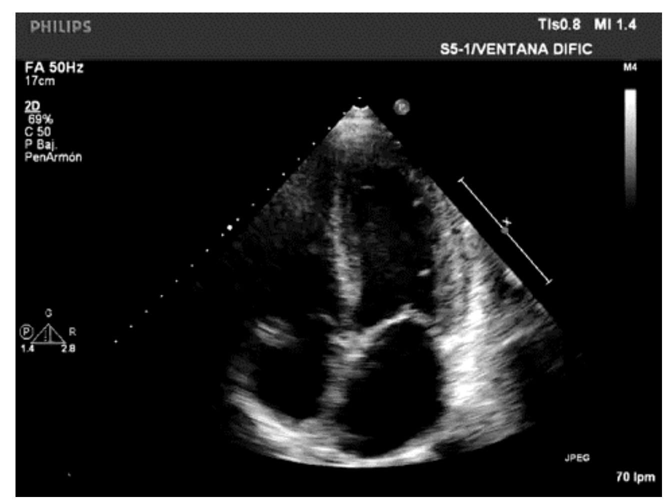


It’s not only about contraction...

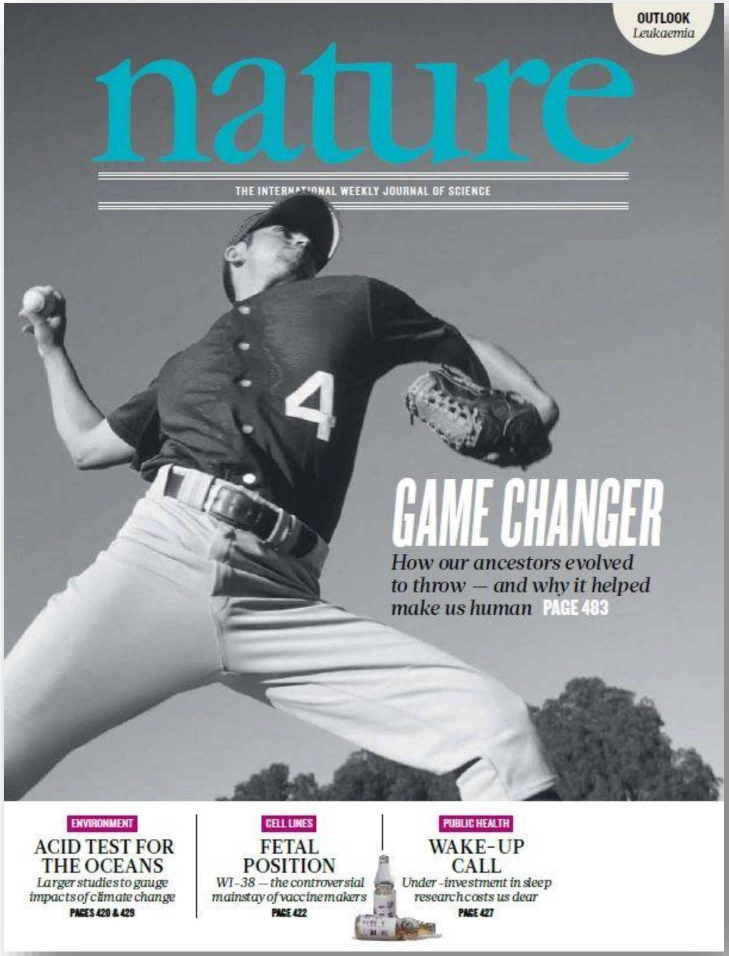
Muscle is elastic!







We need passive elasticity

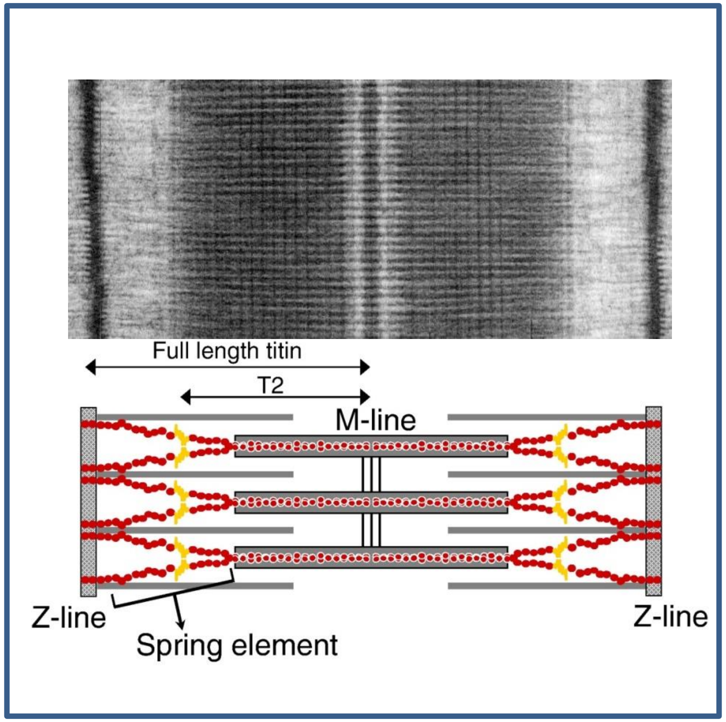


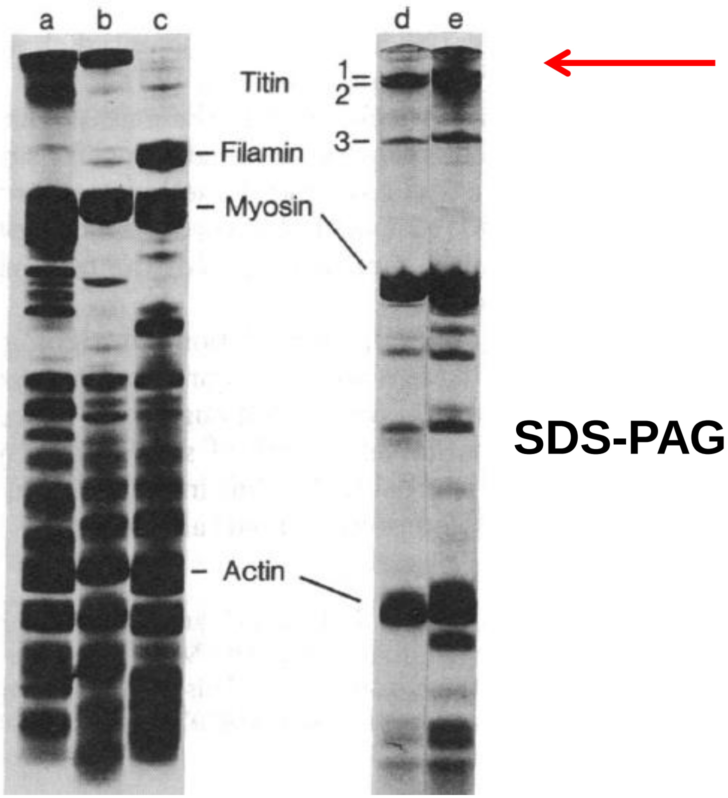
"Compared with other carnivores, [humans] are slow, weak and lack natural weapons such as fangs and claws.

However, [humans] were eating meat at least 2.6 million years (Myr) ago, and were probably hunting large prey 1.9 Myr ago…"

Roach, Venkadesan, Rainbow & Lieberman, Nature 2013

Titin is BIG, a molecular Titan





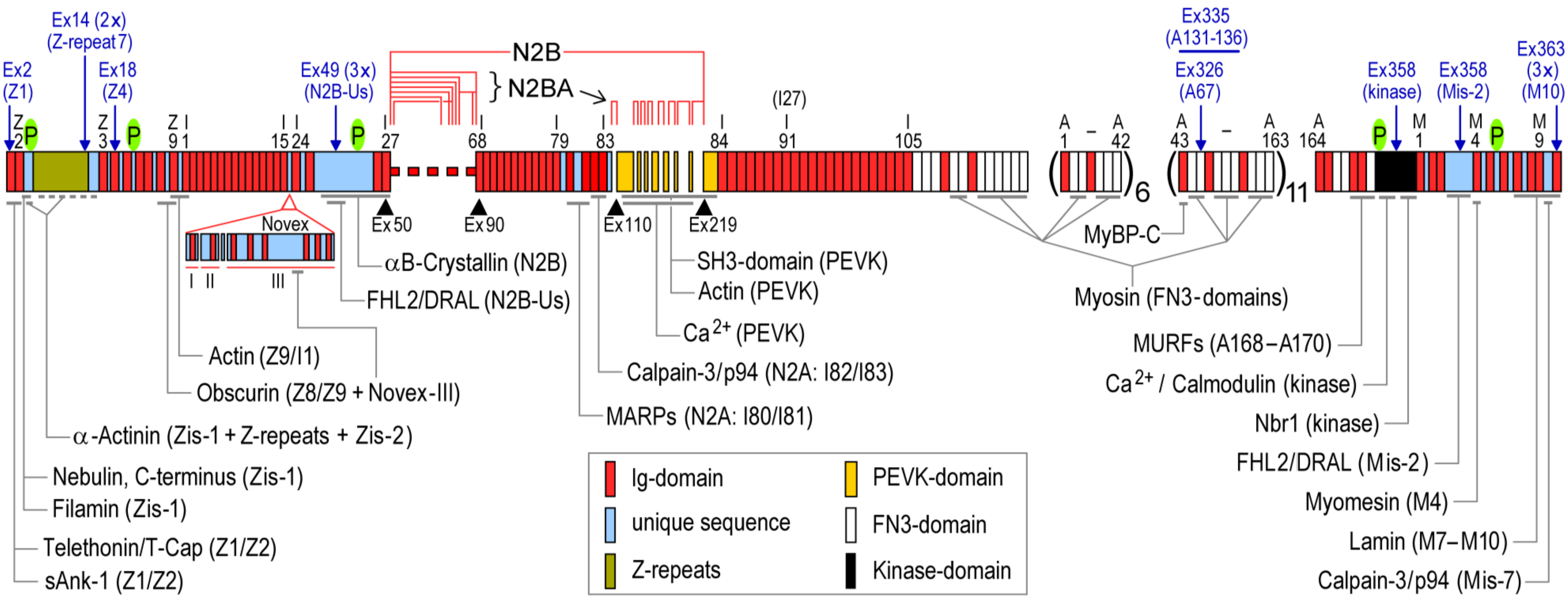
SDS-PAGE (4%)

Wang et al. PNAS 76, 3698 (1979)

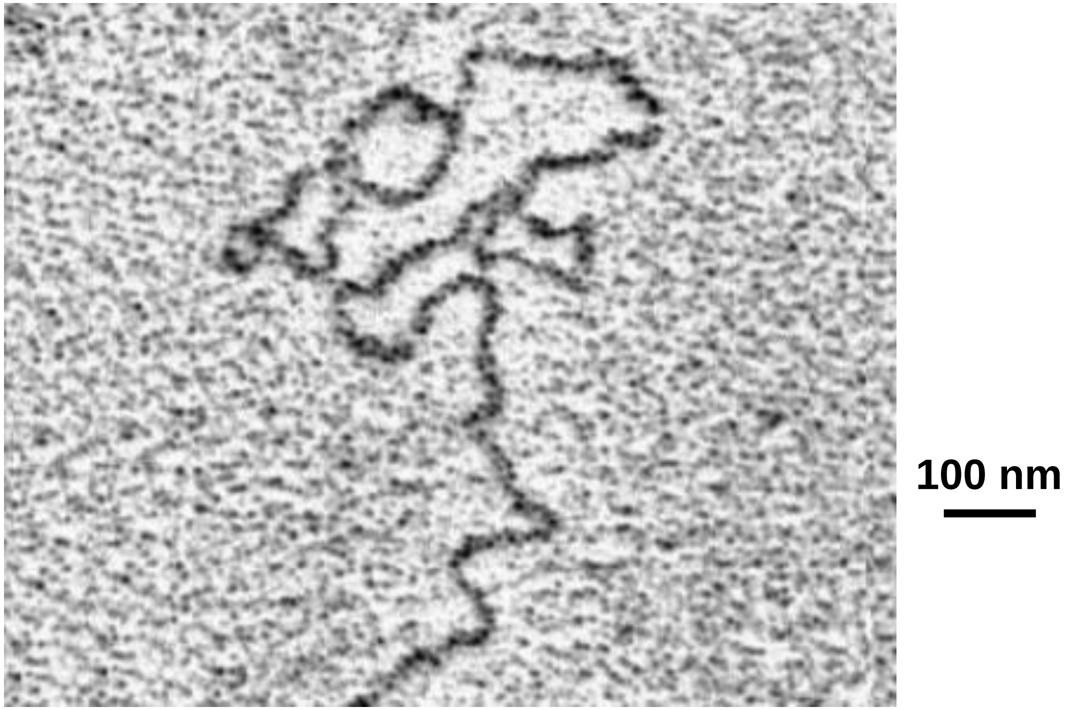
Titin is the largest protein in the human proteome (up to 4 MDa)

The titin gene

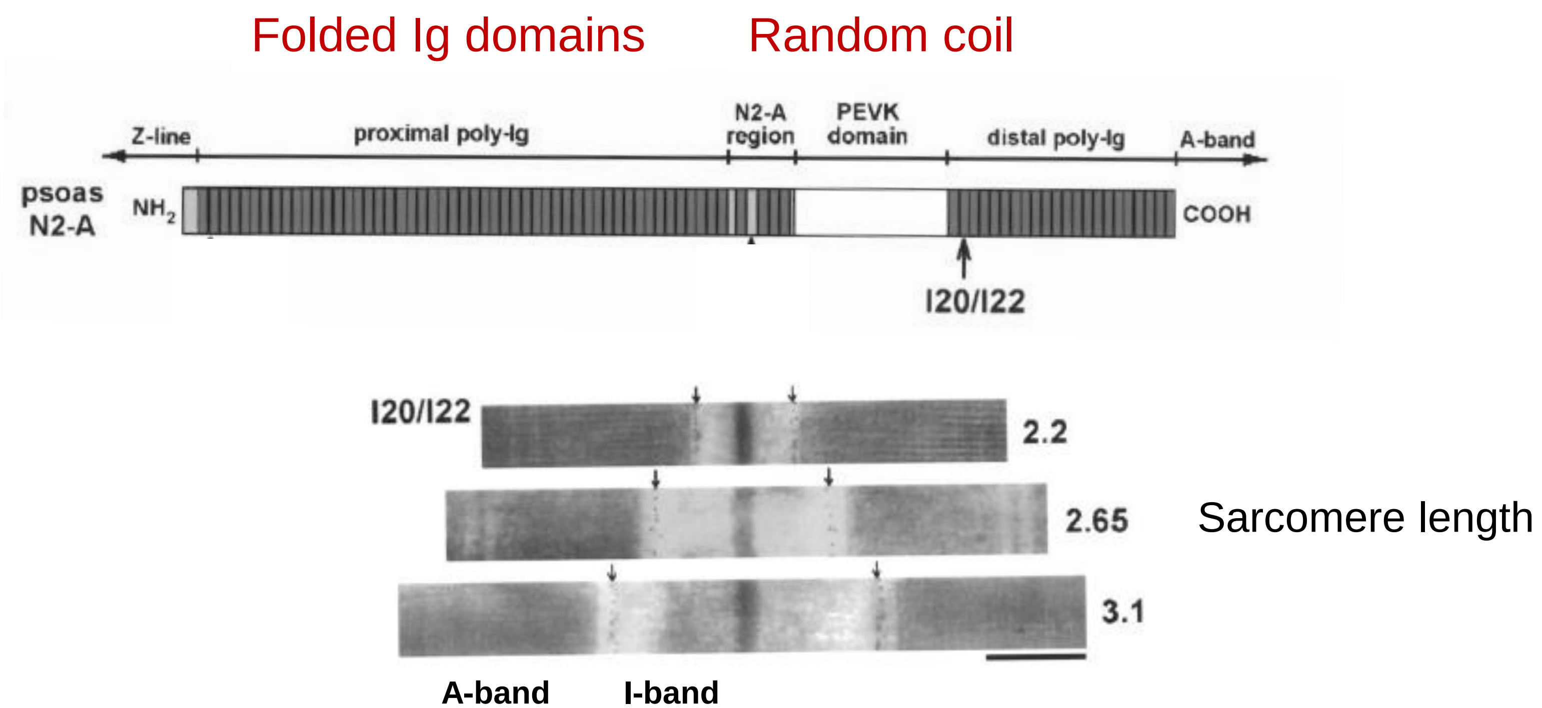
(0.28 Mbp, 363 exons)



Titin has beads-on-a-string appearance

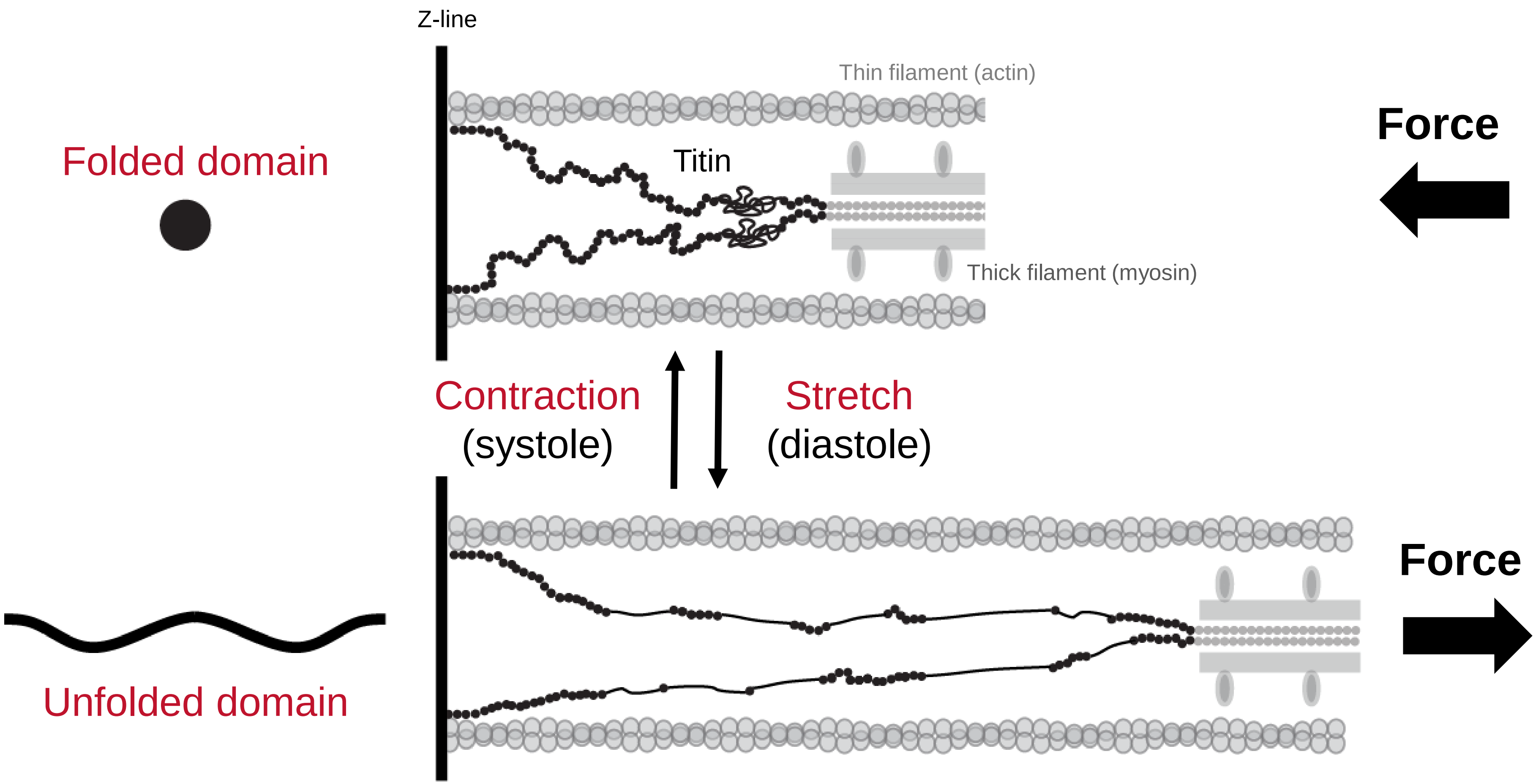


The length of titin changes during contraction/extension cycles

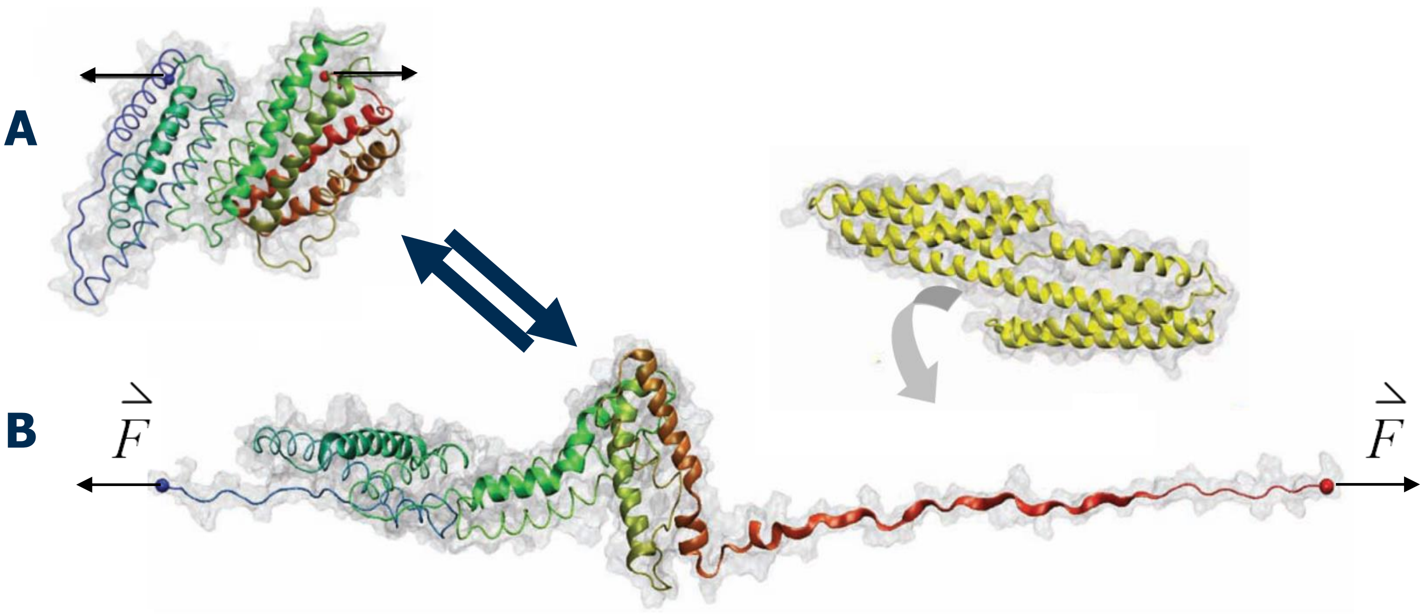


Linke et *al.* J Cell Sci 111, 1567 (1998)

Protein elasticity is determined by protein unfolding/refolding

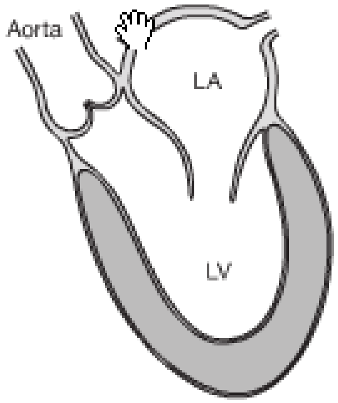


Mechanical forces and exposure of cryptic binding sites

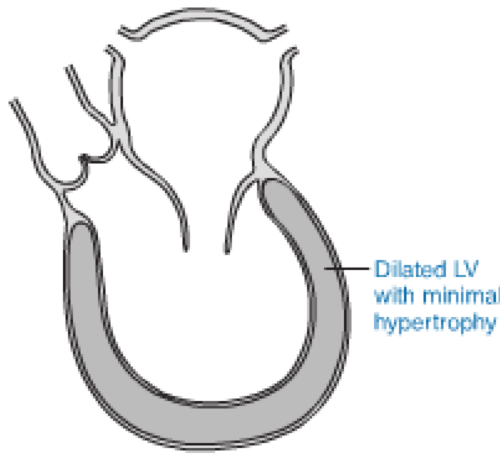


Mechanosensing and mechanotransduction

The mechanics of the myocardium is defective in cardiomyopathies

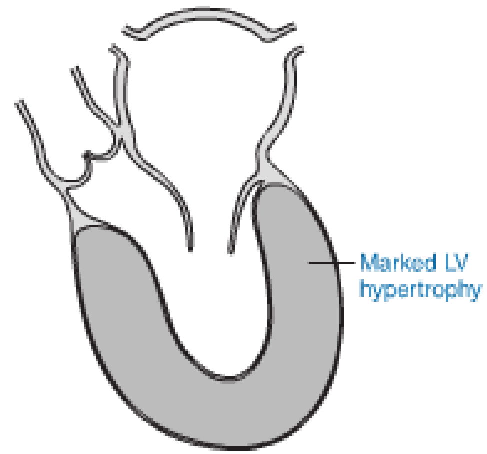


Normal heart



Dilated cardiomyopathy (DCM)

Defective contraction:
impaired systole

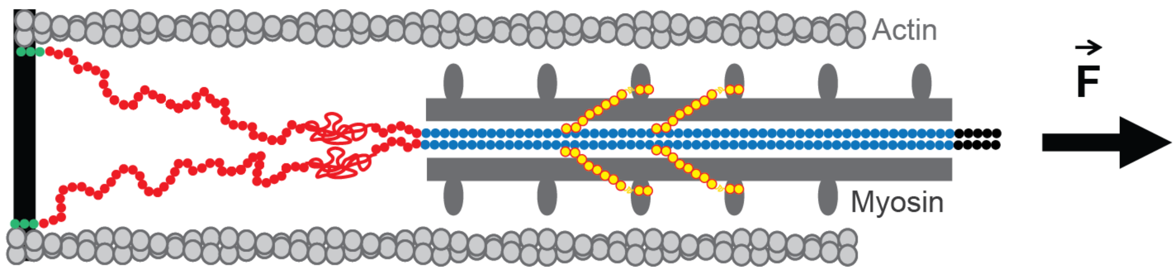


Hypertrophic cardiomyopathy (HCM)

Defective relaxation:
impaired diastole

From “Pathophysiology of Heart Disease”, 5th Edition, Ed. Leonard S. Lilly

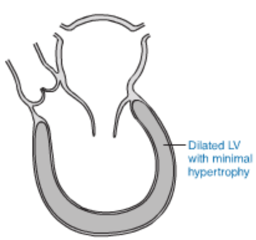
Mutations in sarcomeric proteins lead to familial cardiomyopathy

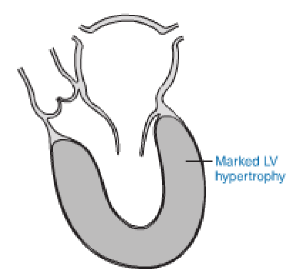


Titin **cMyBPC** **Myosin**

Dilated cardiomyopathy
(~ 25% of cases)

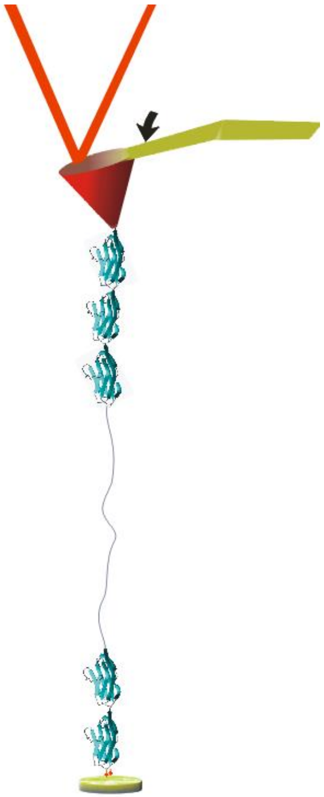
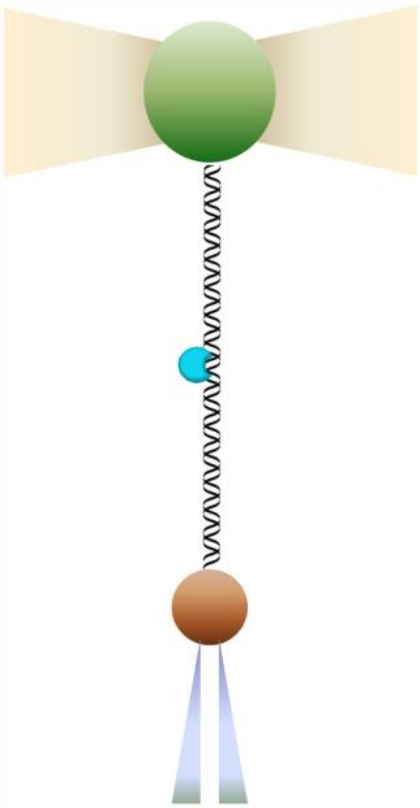
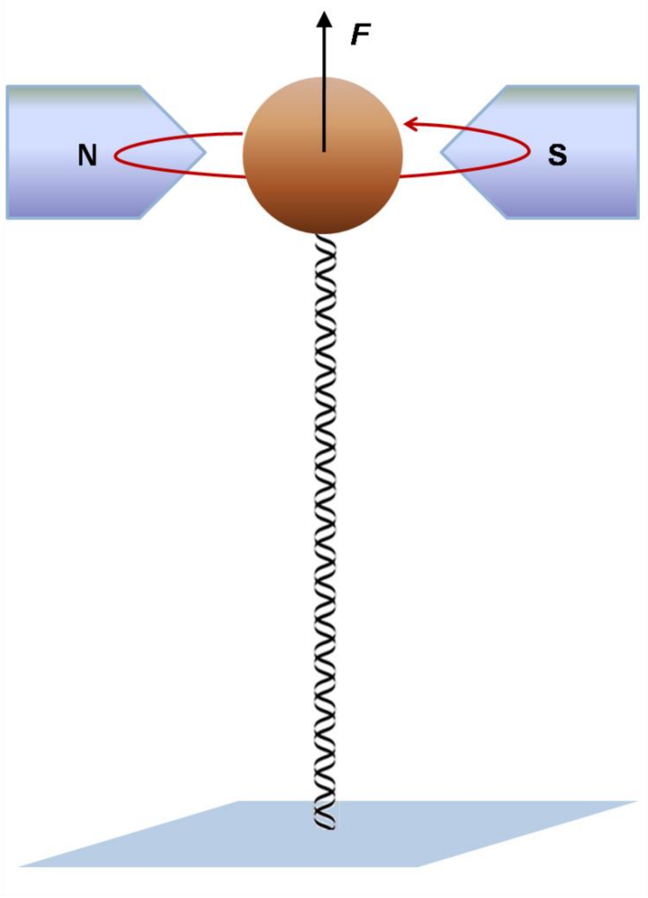
Hypertrophic cardiomyopathy
(~ 70 % of cases)





Genotype to phenotye?

Single-molecule techniques: manipulation

Atomic Force Microscopy(AFM)	Optical Tweezers	Magnetic Tweezers
		

AFM

Force spectroscopy by Atomic Force Microscopy

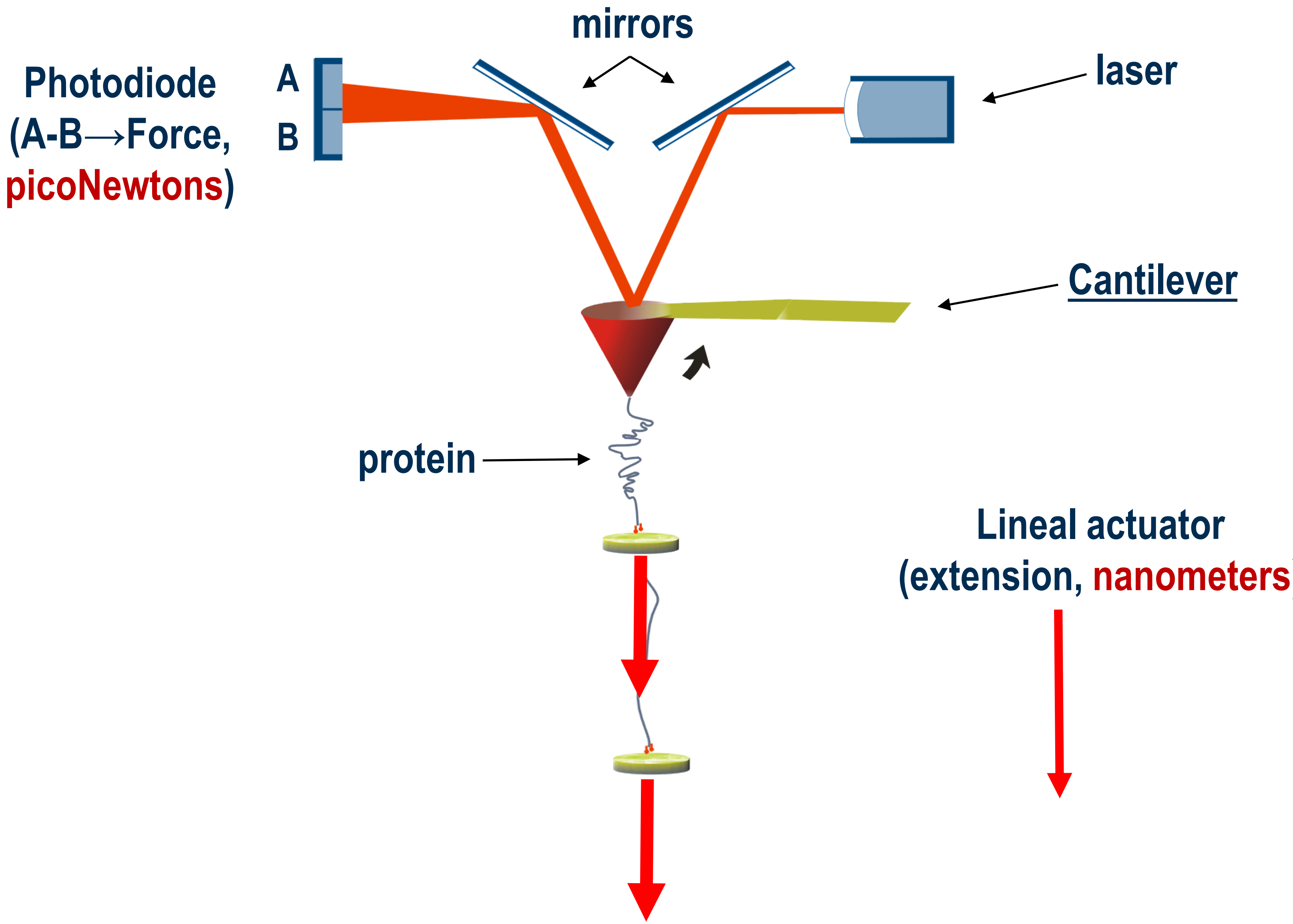
The *pioneer technique* to measure *mechanical properties* of proteins

Reversible Unfolding of Individual Titin Immunoglobulin Domains by AFM

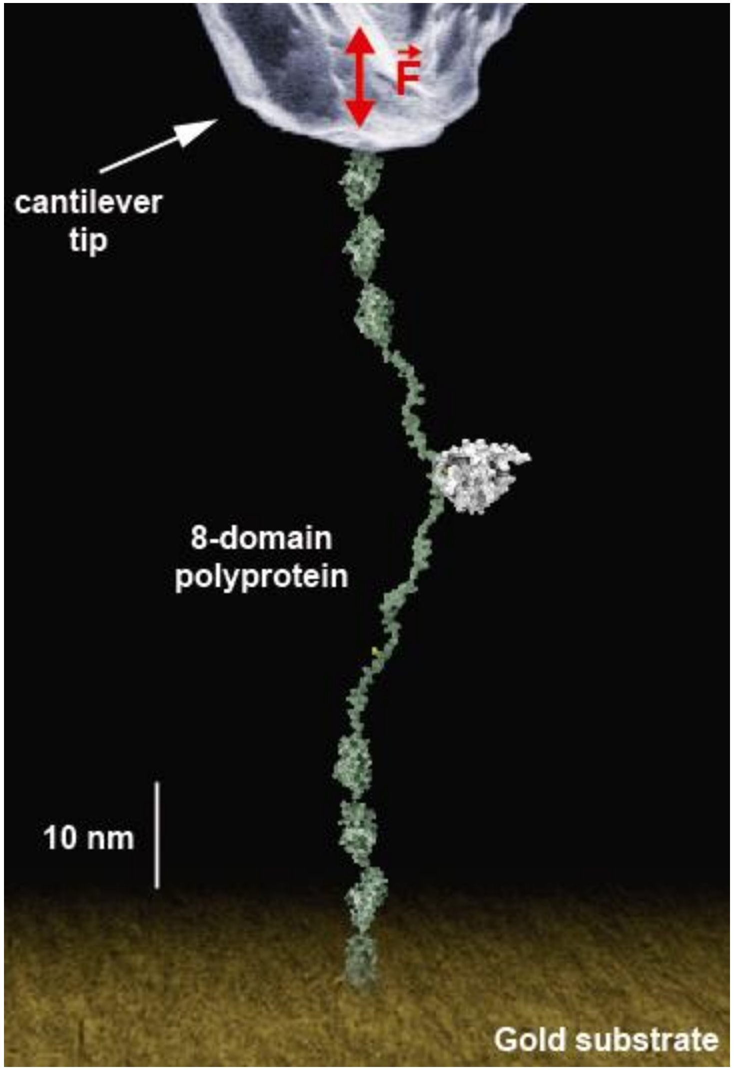
Matthias Rief, Mathias Gautel, Filipp Oesterhelt, Julio M. Fernandez, Hermann E. Gaub*

Science (1997) 276, 1109

Constant-velocity experiments: *force-extension*

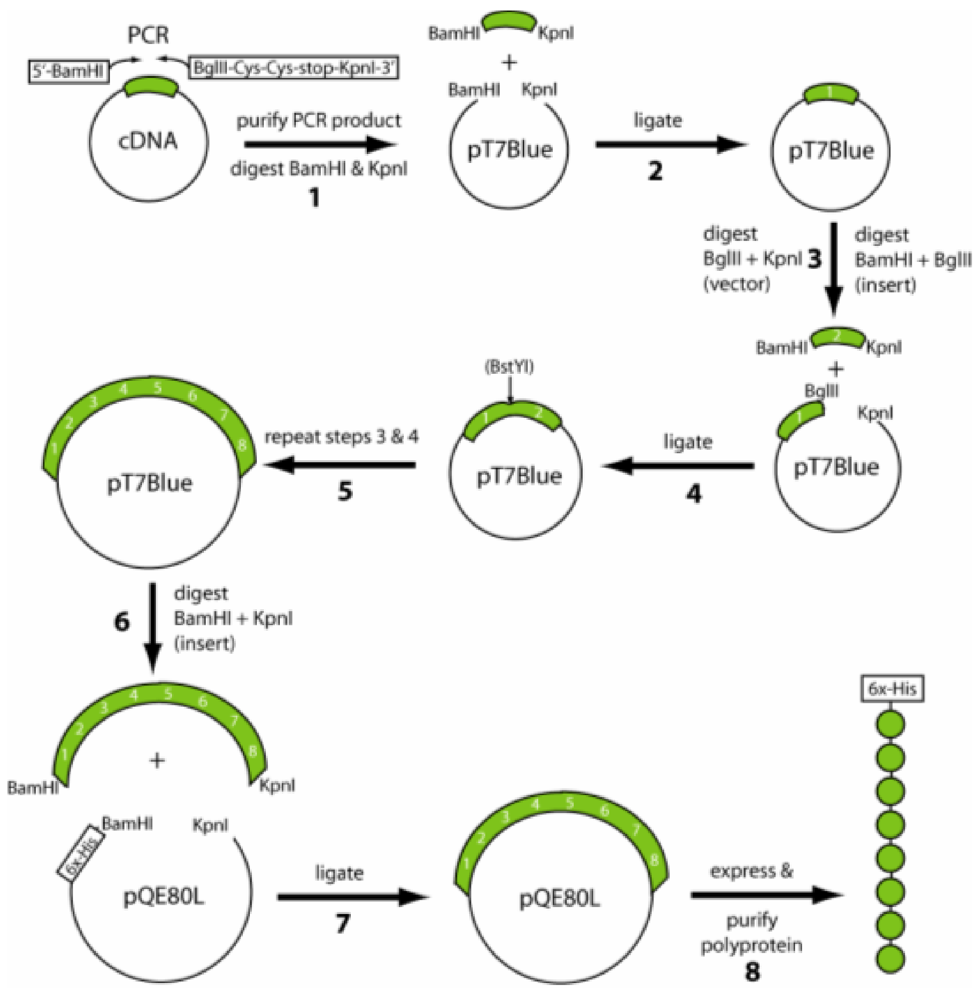


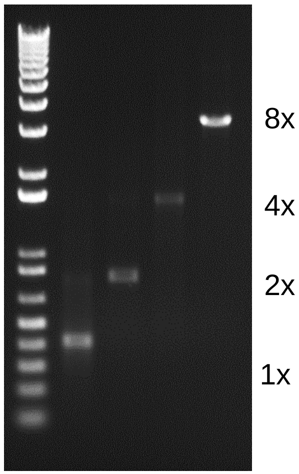
A more realistic view of an AFM pulling experiment

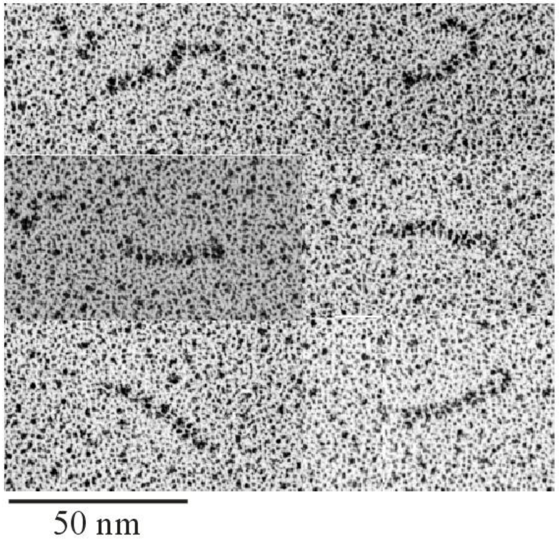


Polyproteins are *“minititins”*

Polyprotein engineering for force spectroscopy

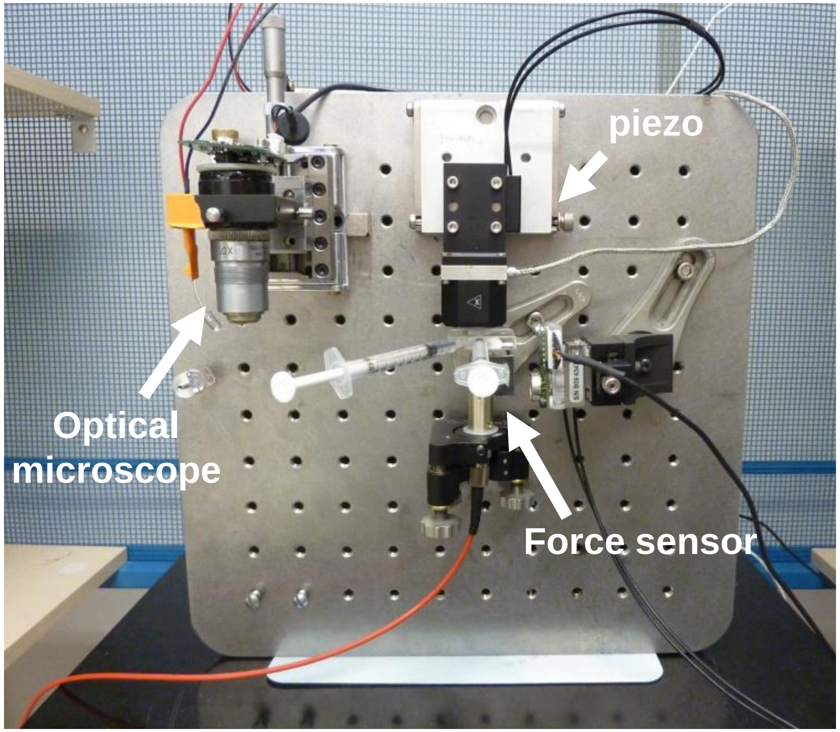




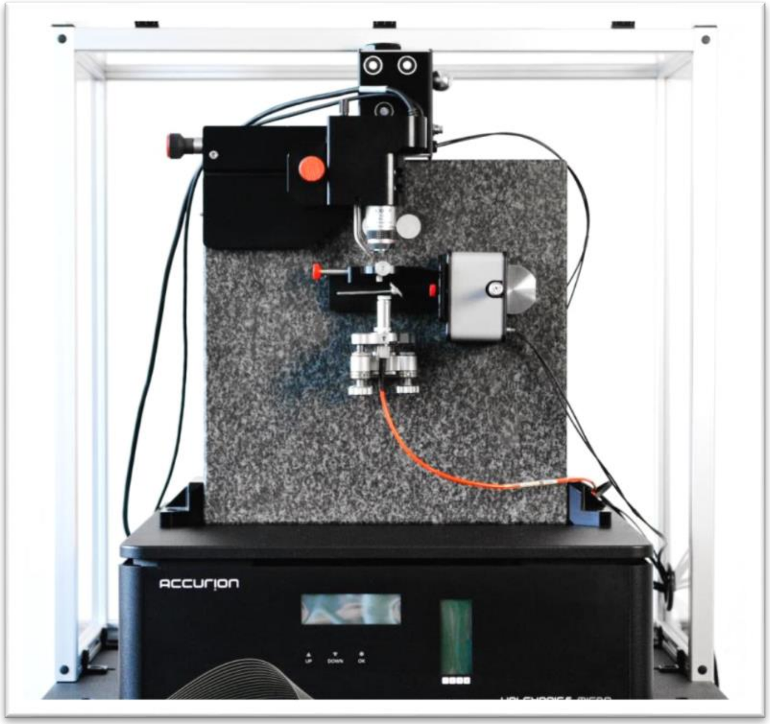


Atomic Force Microscopes/Spectrometers

Home-made AFM

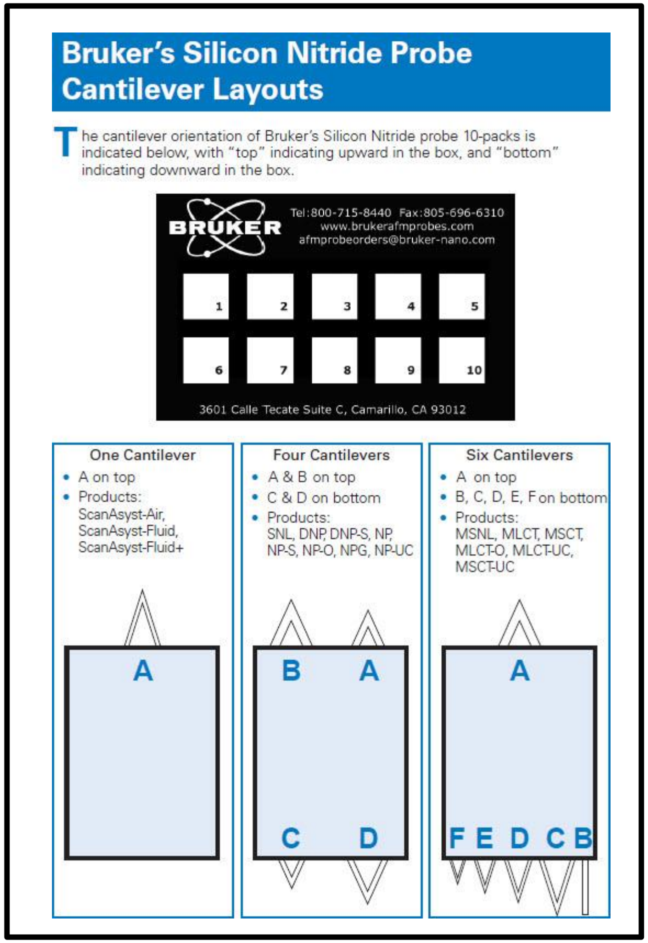


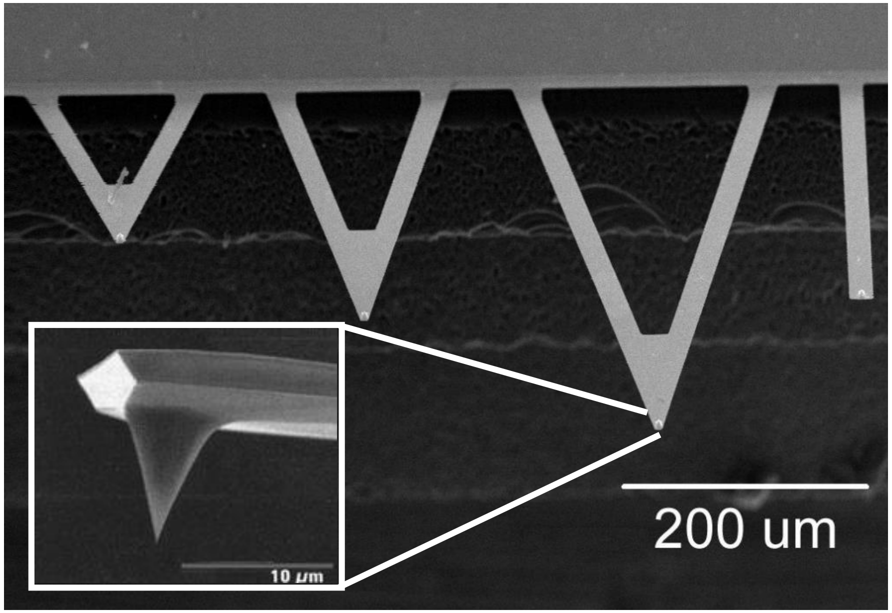
Commercial AFM



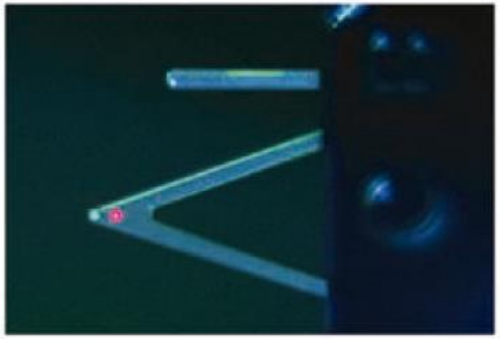
At CNIC

AFM cantilevers for single-molecule experiments



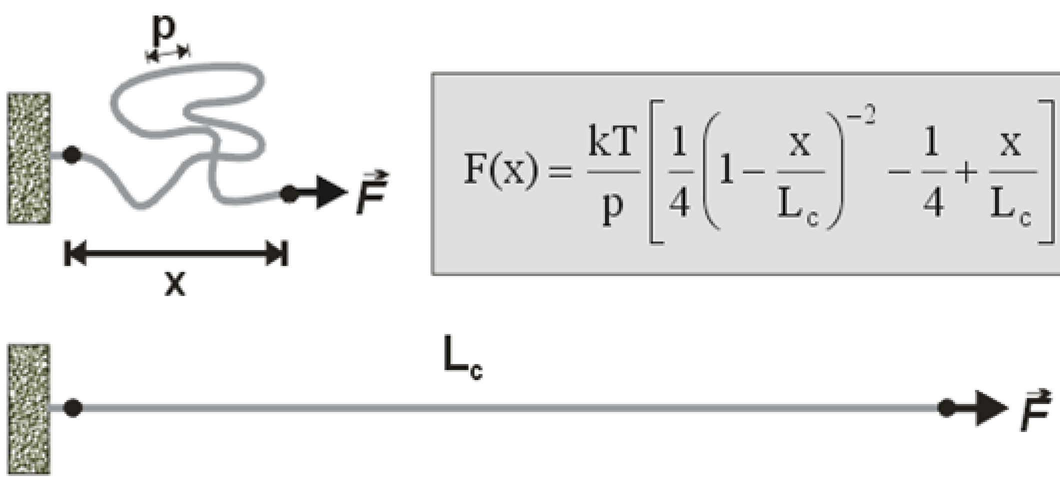


Spring constant: 5-20 pN/nm



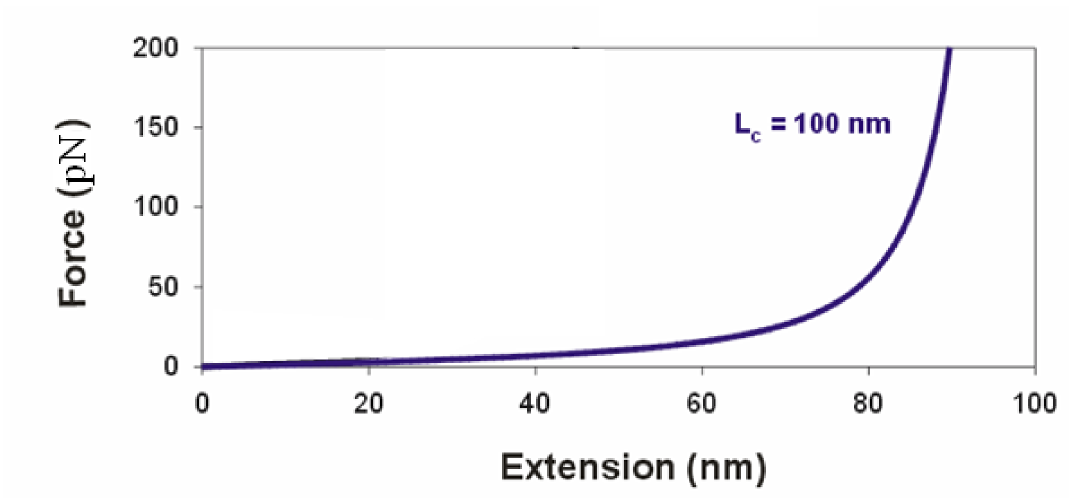
Enfoque del láser

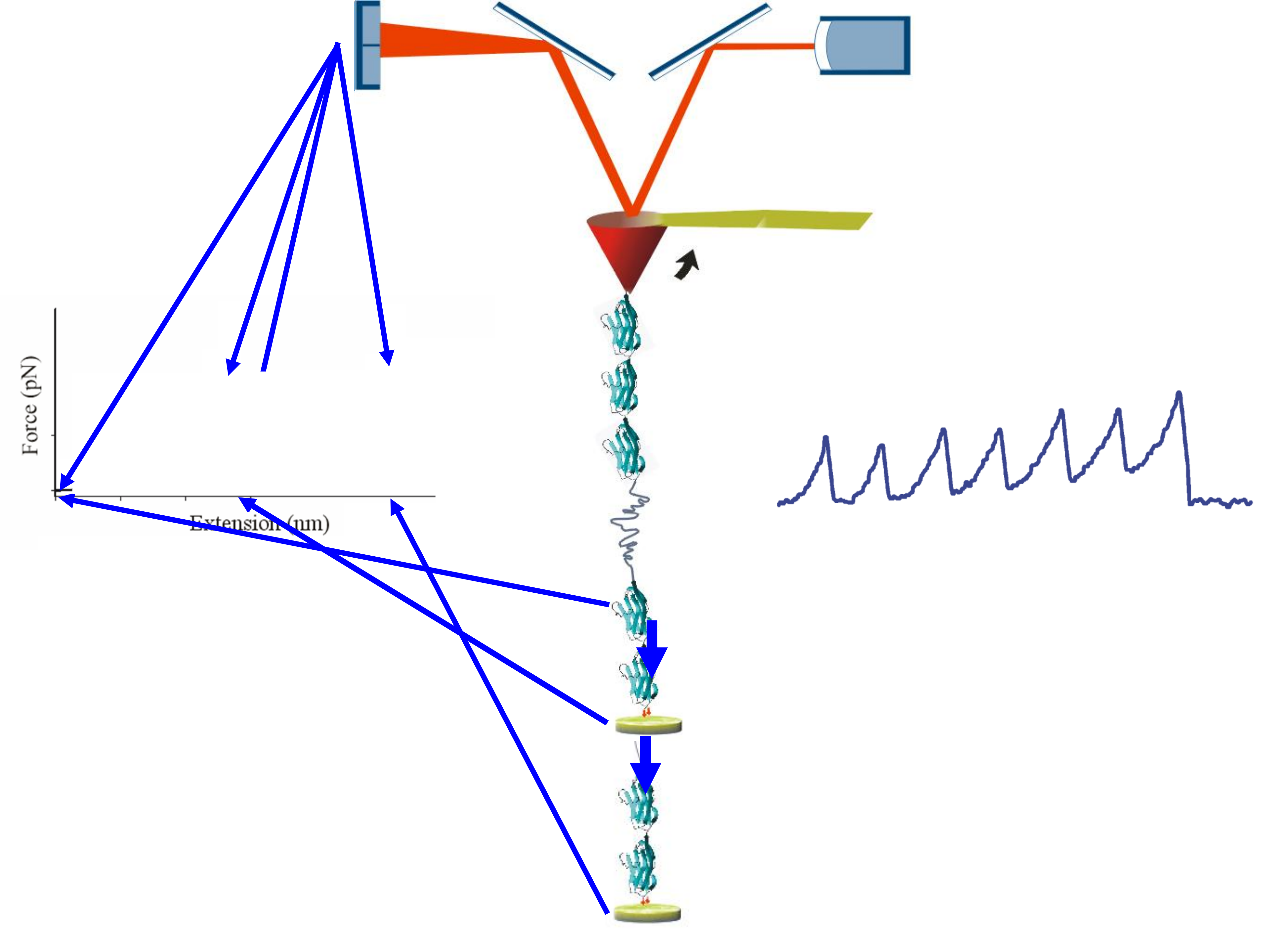
Worm-like chain model of polymer elasticity



$$F(x) = \frac{kT}{p}\left[\frac{1}{4}\left(1 - \frac{x}{L_c}\right)^{-2} - \frac{1}{4} + \frac{x}{L_c}\right]$$

- x : extension
- L_c: contour length
  (length at infinite force)
- p: persistence length
  (~internal flexibility)

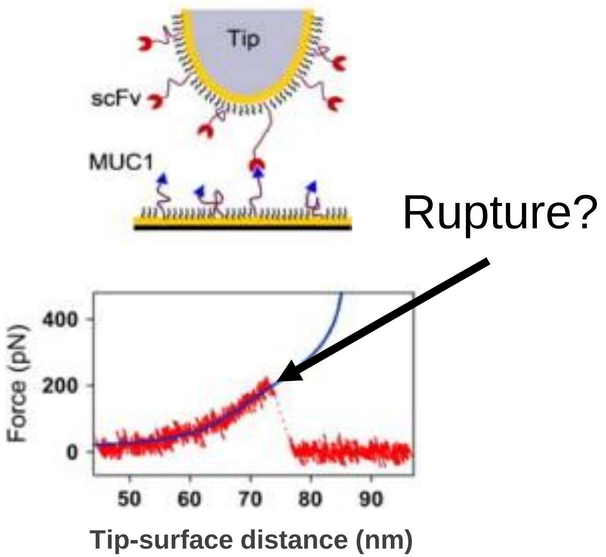




Force (pN)
Extension (nm)

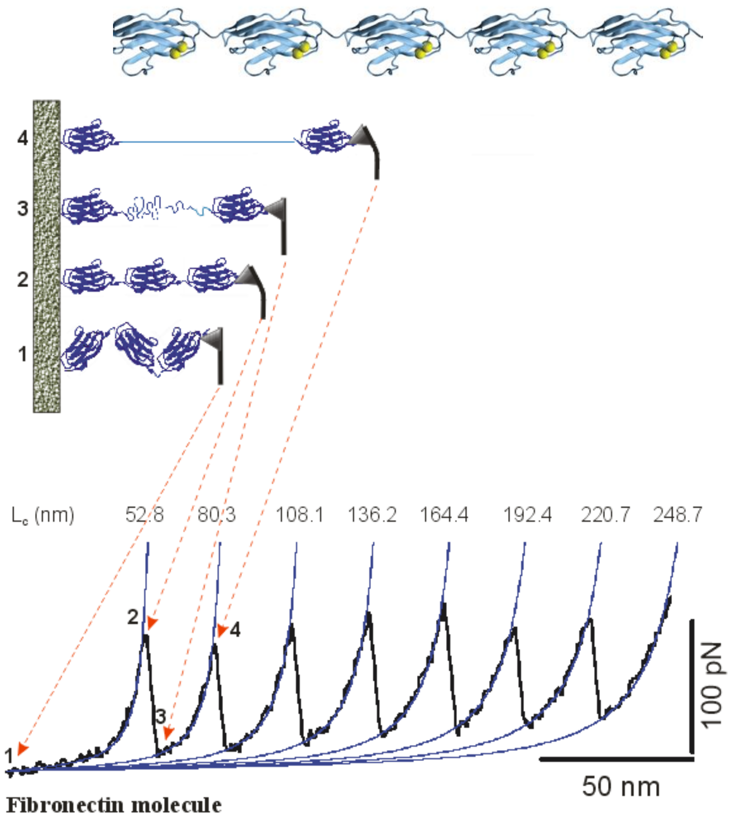
The importance of fingerprinting single-molecule data

Bond rupture



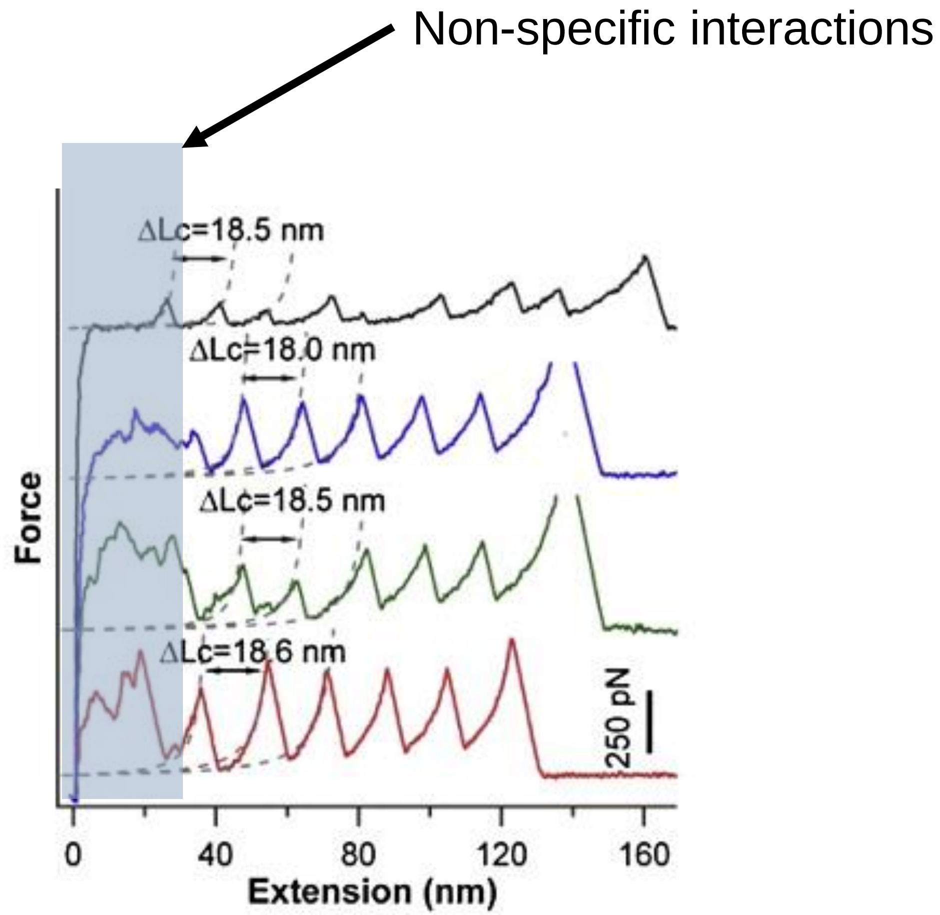
Length

Protein unfolding



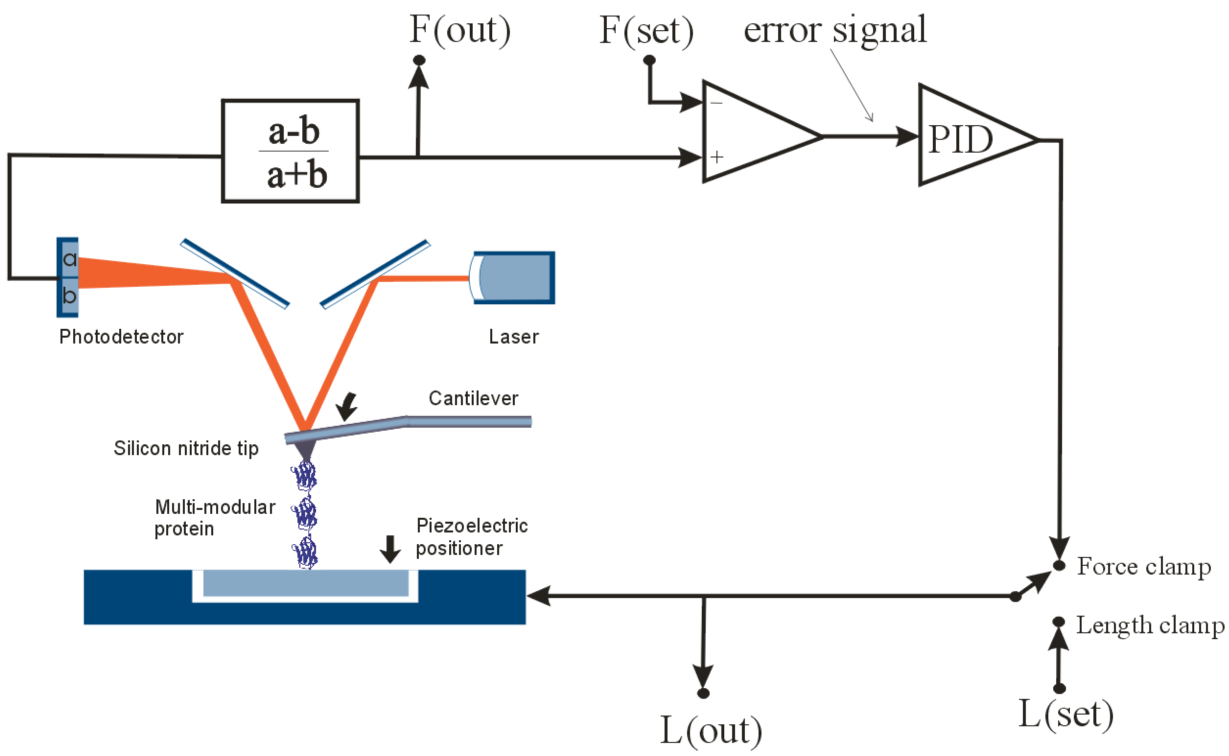
Repetitive recording (sawtooth)

Non-specific interactions happen close to the surface

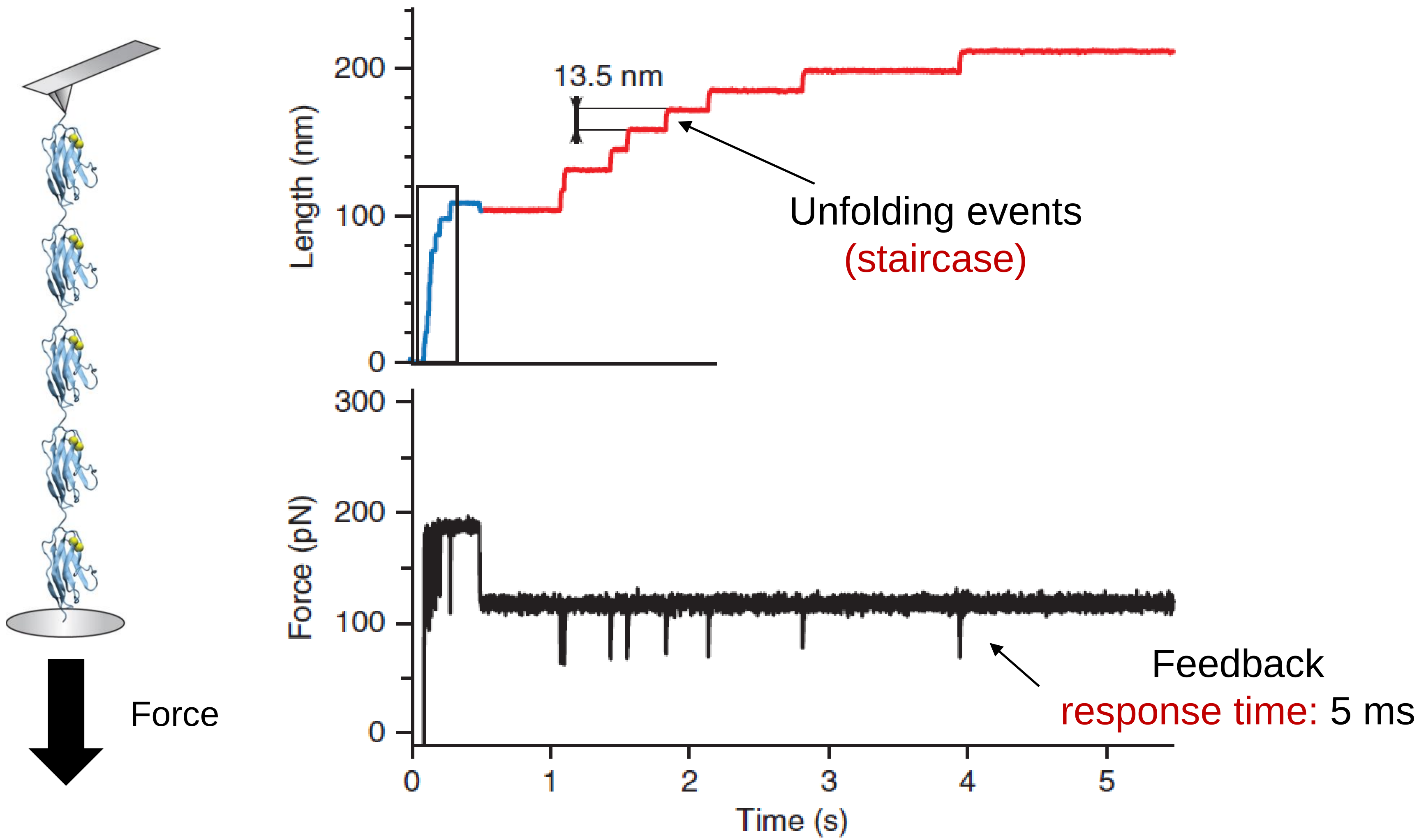


Constant force experiments: *force-clamp*

- Better approach to determine force dependencies
- Feedback systems to keep the force at a predefined set point

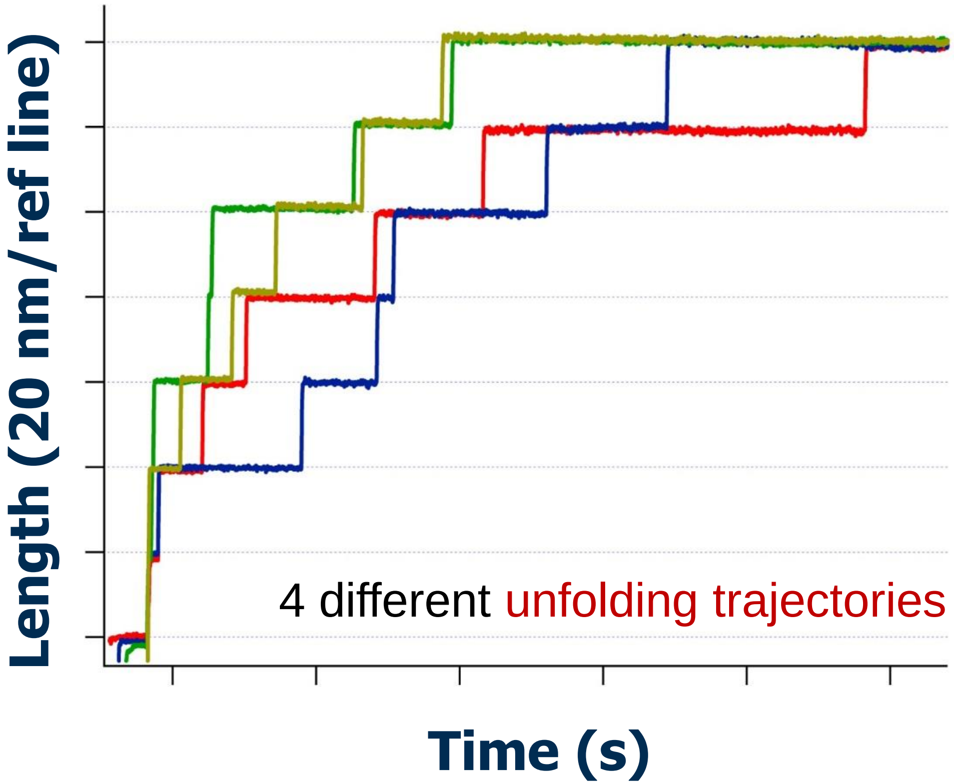


A force-clamp experimental trace



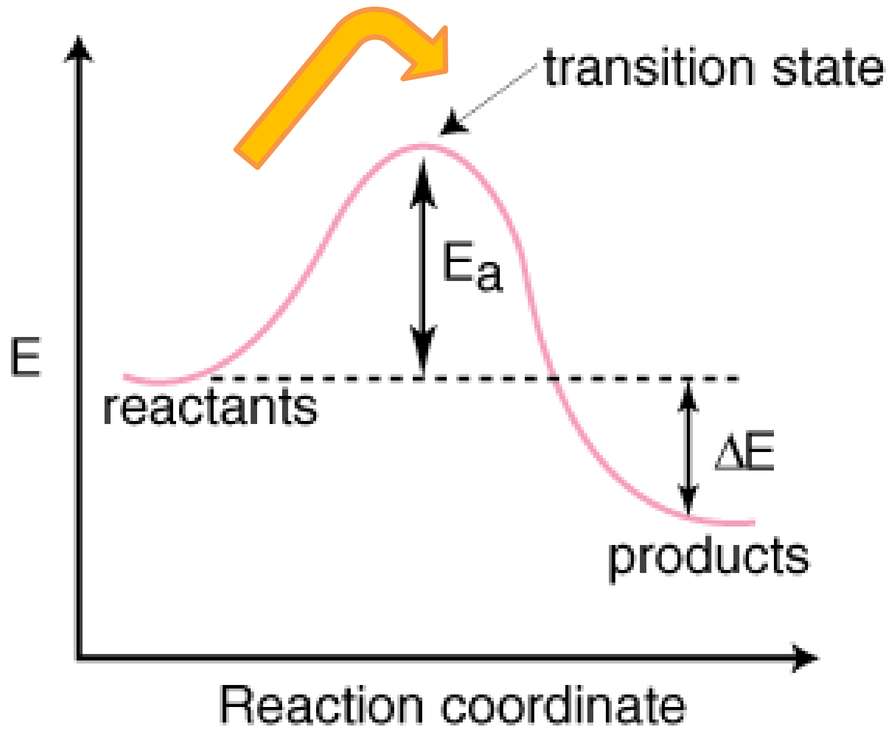
New mindset:
Single-molecule events are stochastic

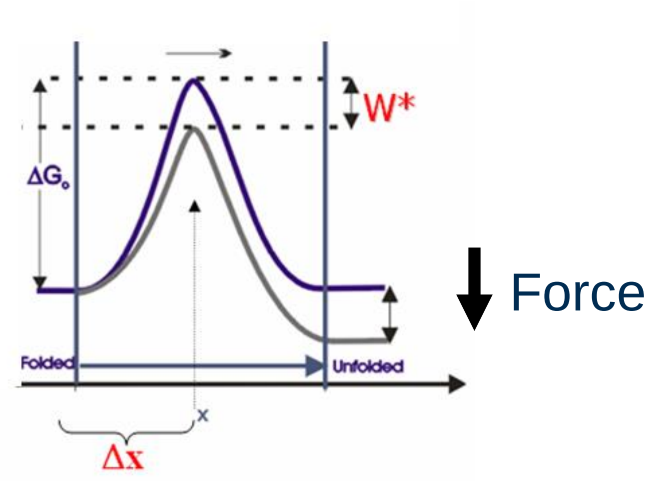
SINGLE-MOLECULE *Vs.* BULK



Variant	Melting temperature
WT	66.3
D122Y	50.4
G130V	43.2
G137V	46.0
I154F	50.7
W155R	61.4

Crossing of energy barriers at the single-molecule level

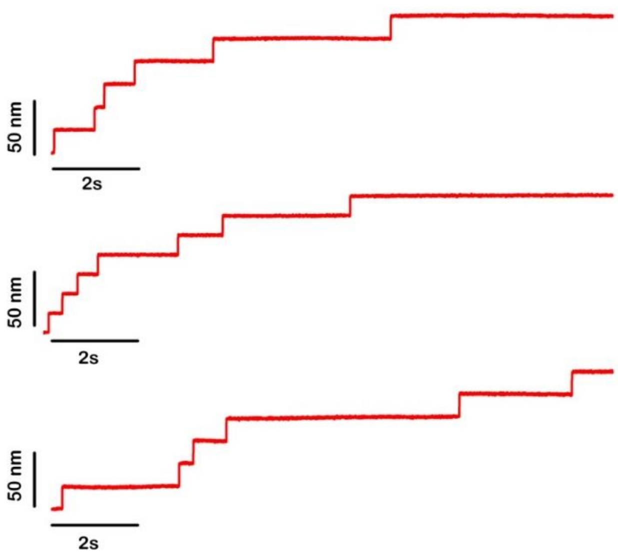




$$k_u = k_u^0 e^{F\Delta x / kT}$$

Measuring kinetics of mechanical protein unfolding

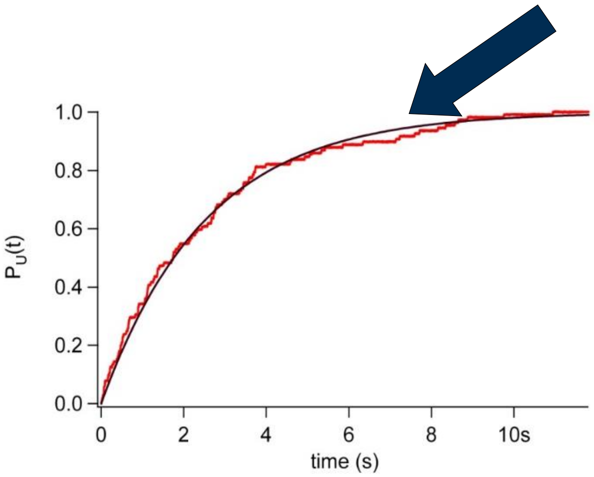
$$N \xrightarrow{k(F)} U$$



F = 140 pN

Average multiple single-molecule trajectories (>20)

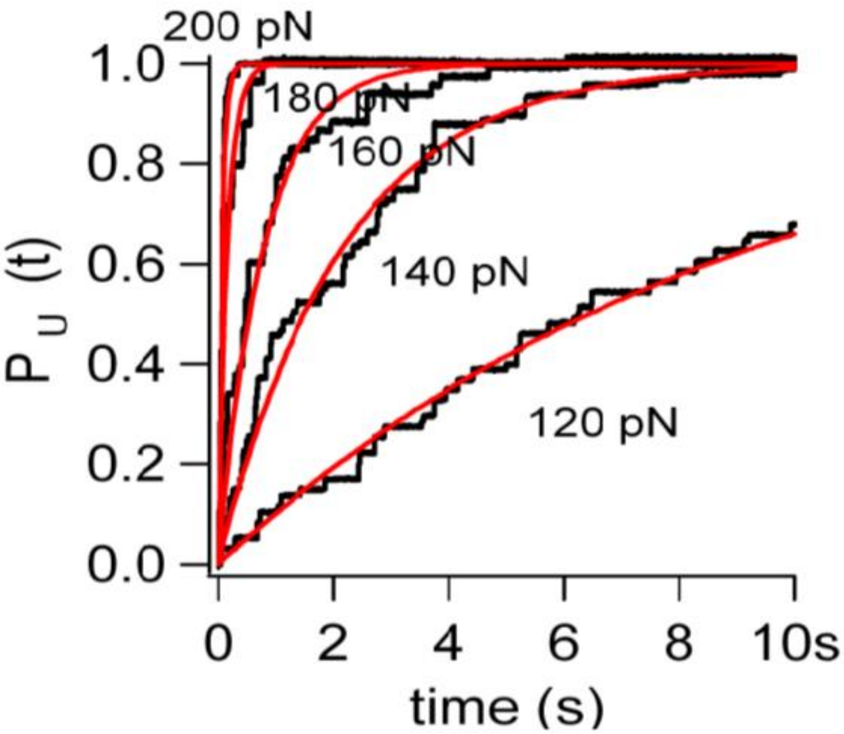
k (F)

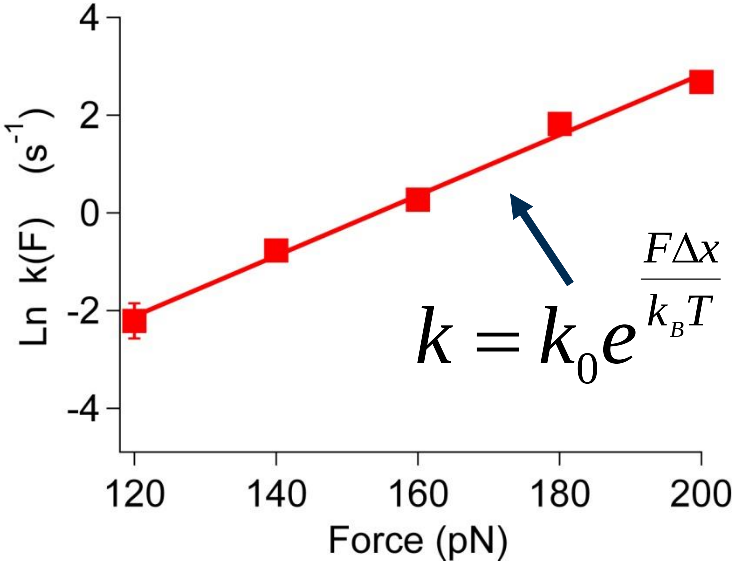


Fit to a simple exponential function

$$P(U) = 1 - e^{-k(F)\cdot t}$$

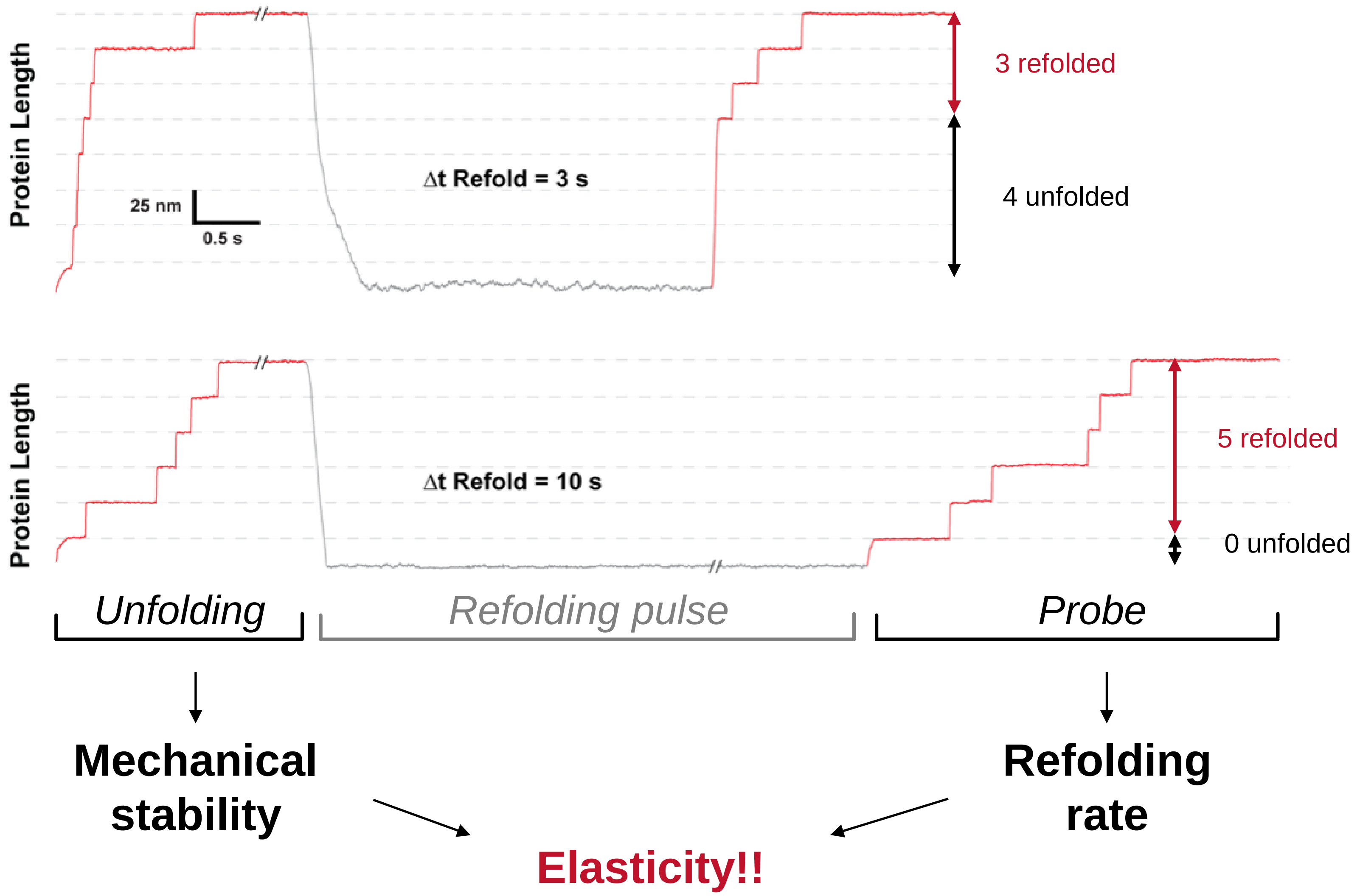
Force-dependent mechanical unfolding





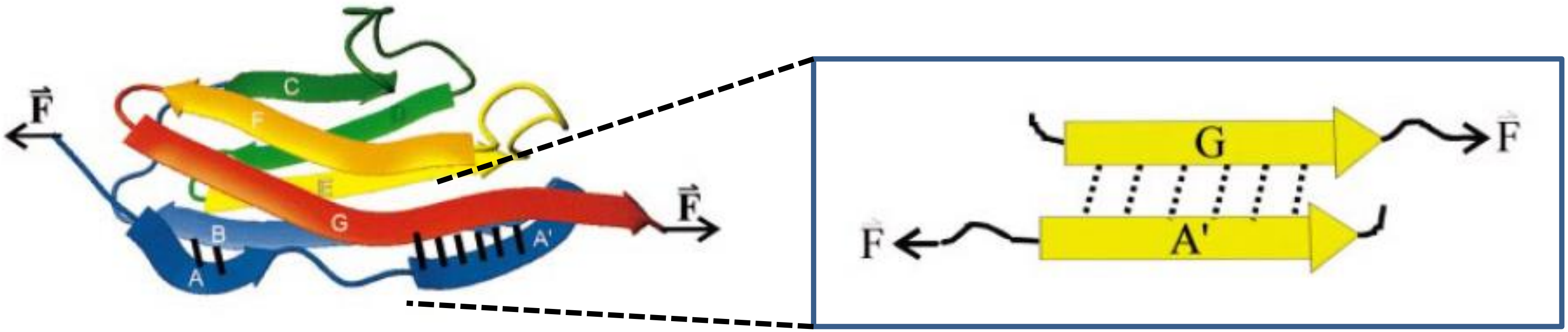
The higher the force, the faster proteins unfold

Mechanical refolding by AFM

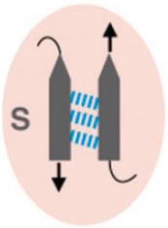


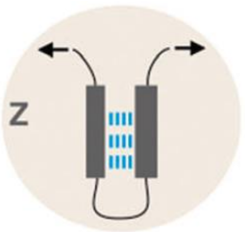
Molecular determinants of the mechanical stability of proteins

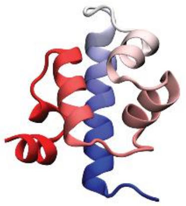
Mechanical transition state



Mechanical stability: (β-shearing > β-unzipping > alpha)

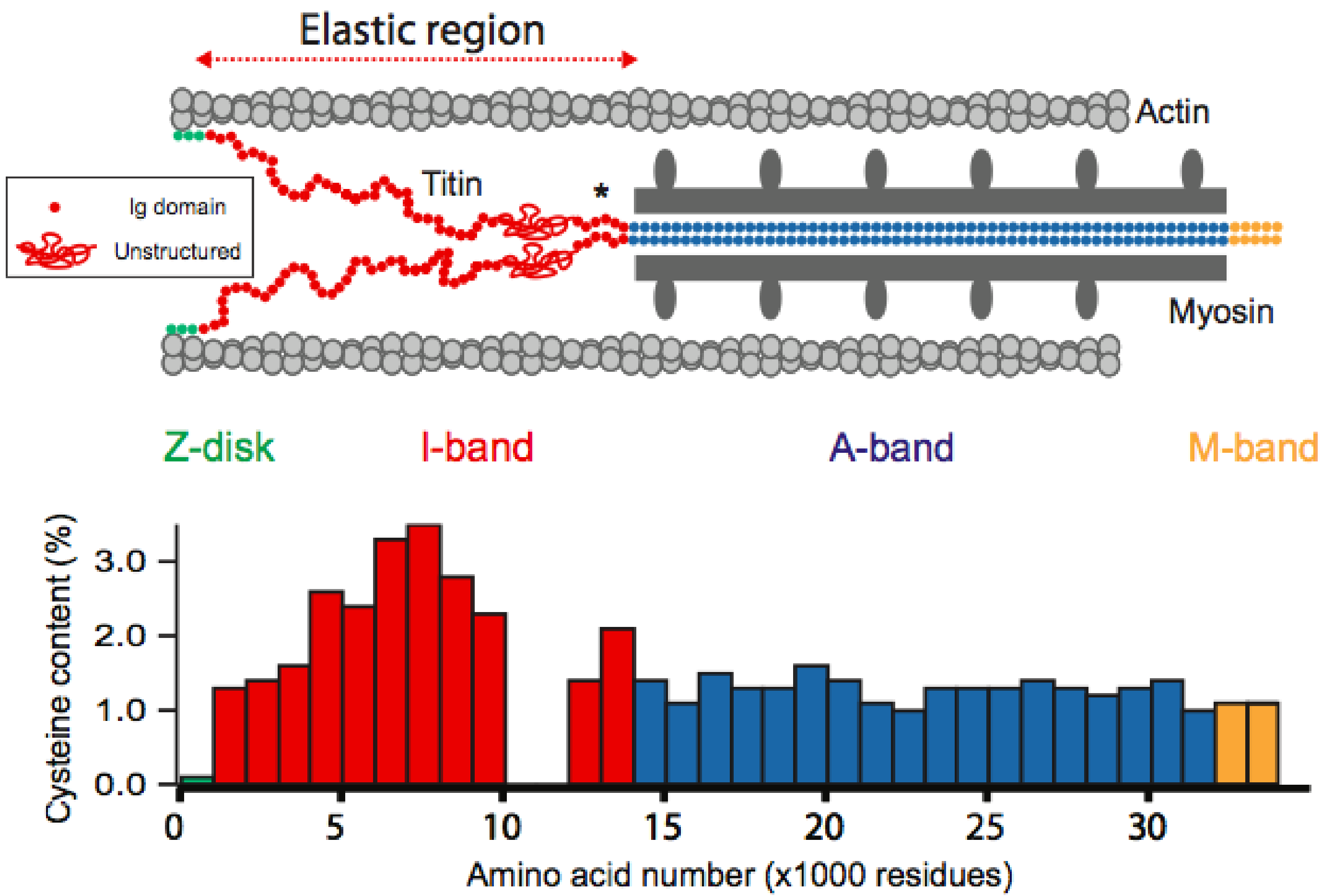






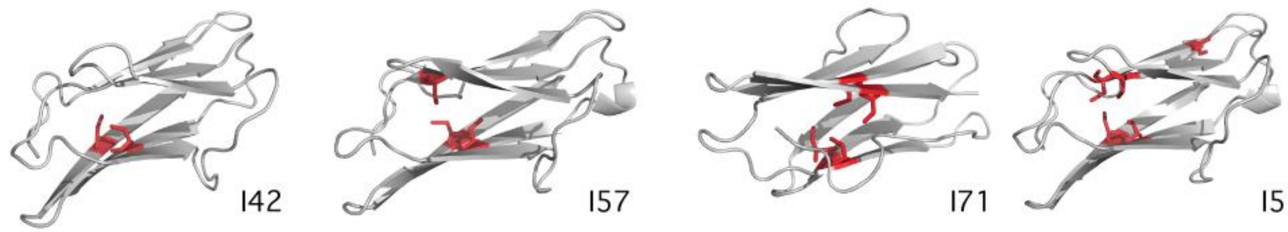
Folding?

An example of single-molecule experiments informing about biology: Thiol chemistry controlling titin elasticity

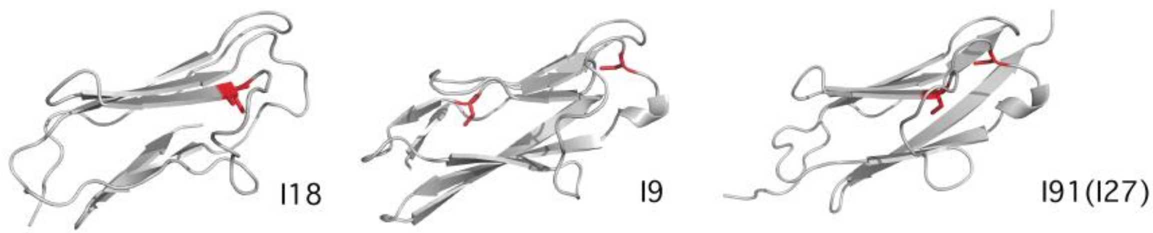


Titin’s buried (cryptic) cysteines

Paired



Unpaired



Redox posttranslational modifications in muscle

Xanthine oxidase
NAD(P)H oxidase

ROS (superoxide, H_2O_2)

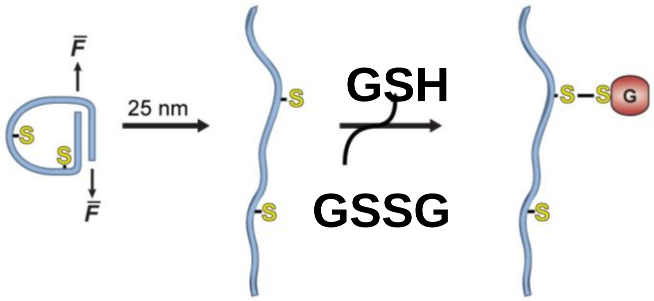
Oxidative modification of cysteines in titin

What's the functional relevance?

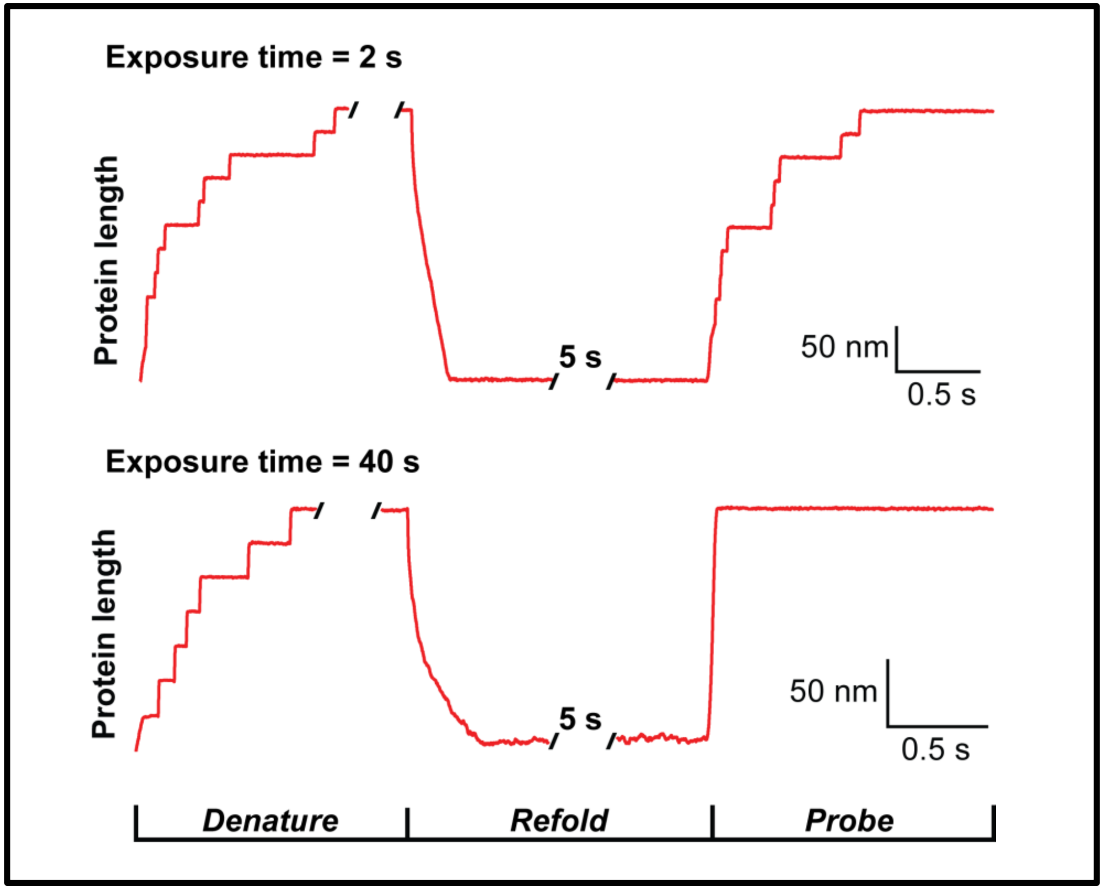
NO

Nitric oxide synthase

S-glutathionylation inhibits protein folding



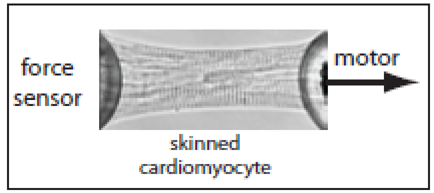
I27 polyprotein



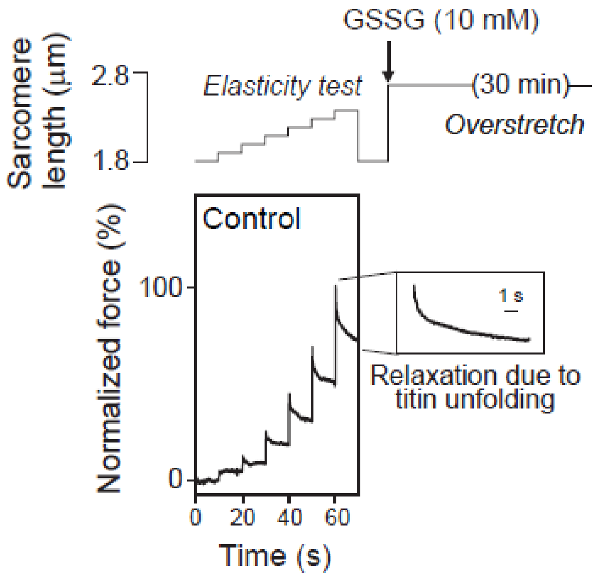
Inhibition of folding

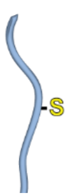
Softening of the tissue

The elasticity of cardiomyocytes is modulated by S-glutathionylation of titin's cryptic cysteines



In collaboration with Nazha Hamdani and Wolfgang Linke (Bochum University, Germany)

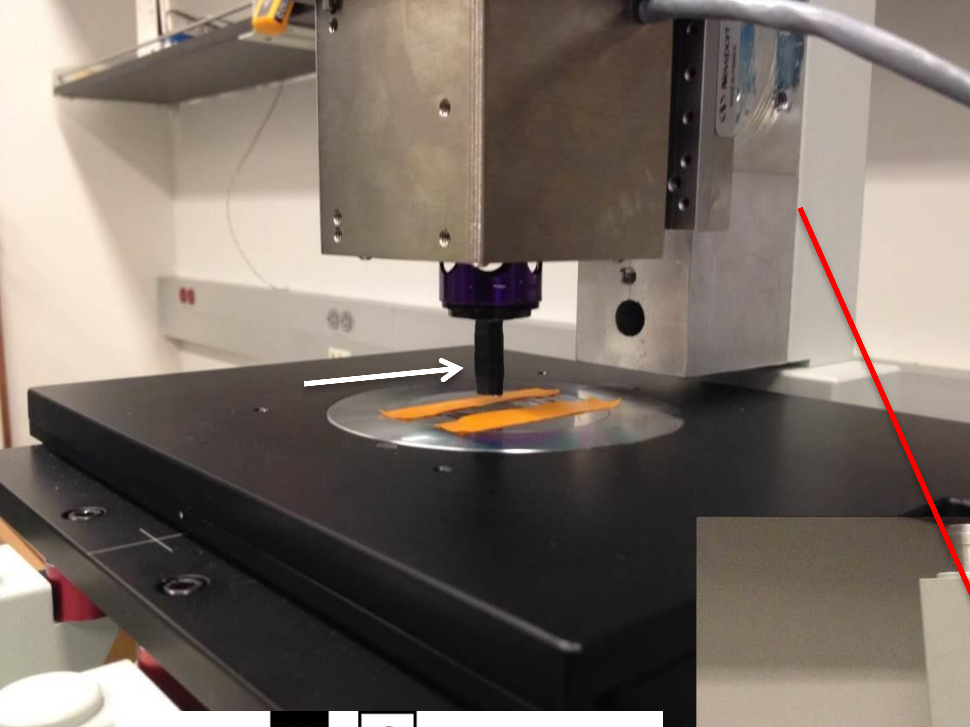




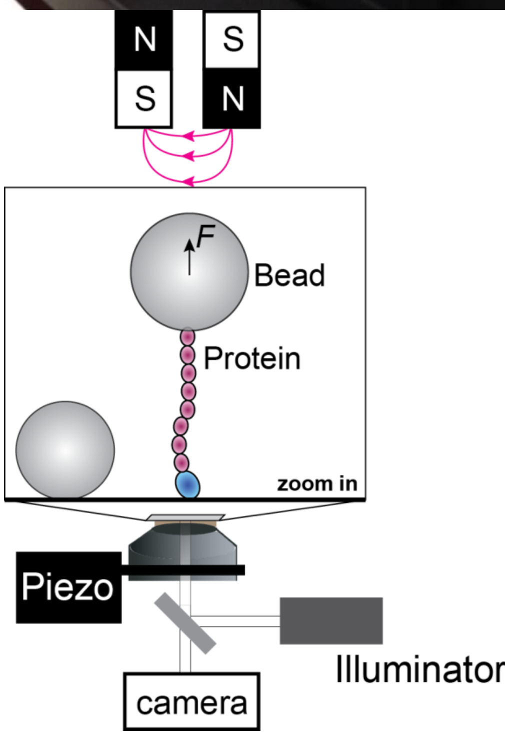
Alegre-Cebollada*, Kosuri* *et al.*
Cell,156, 1235 (2014)

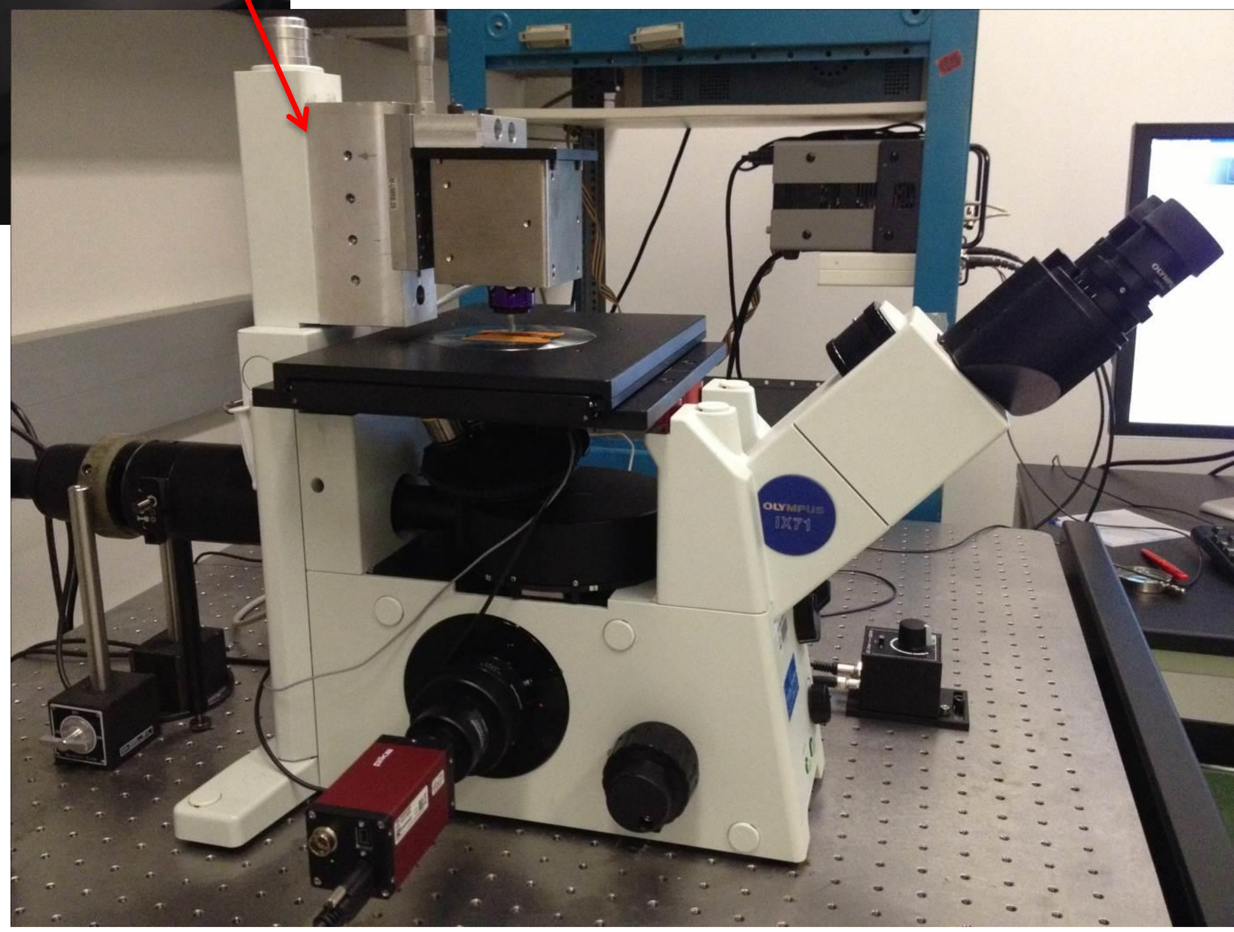
MT

Magnetic tweezers

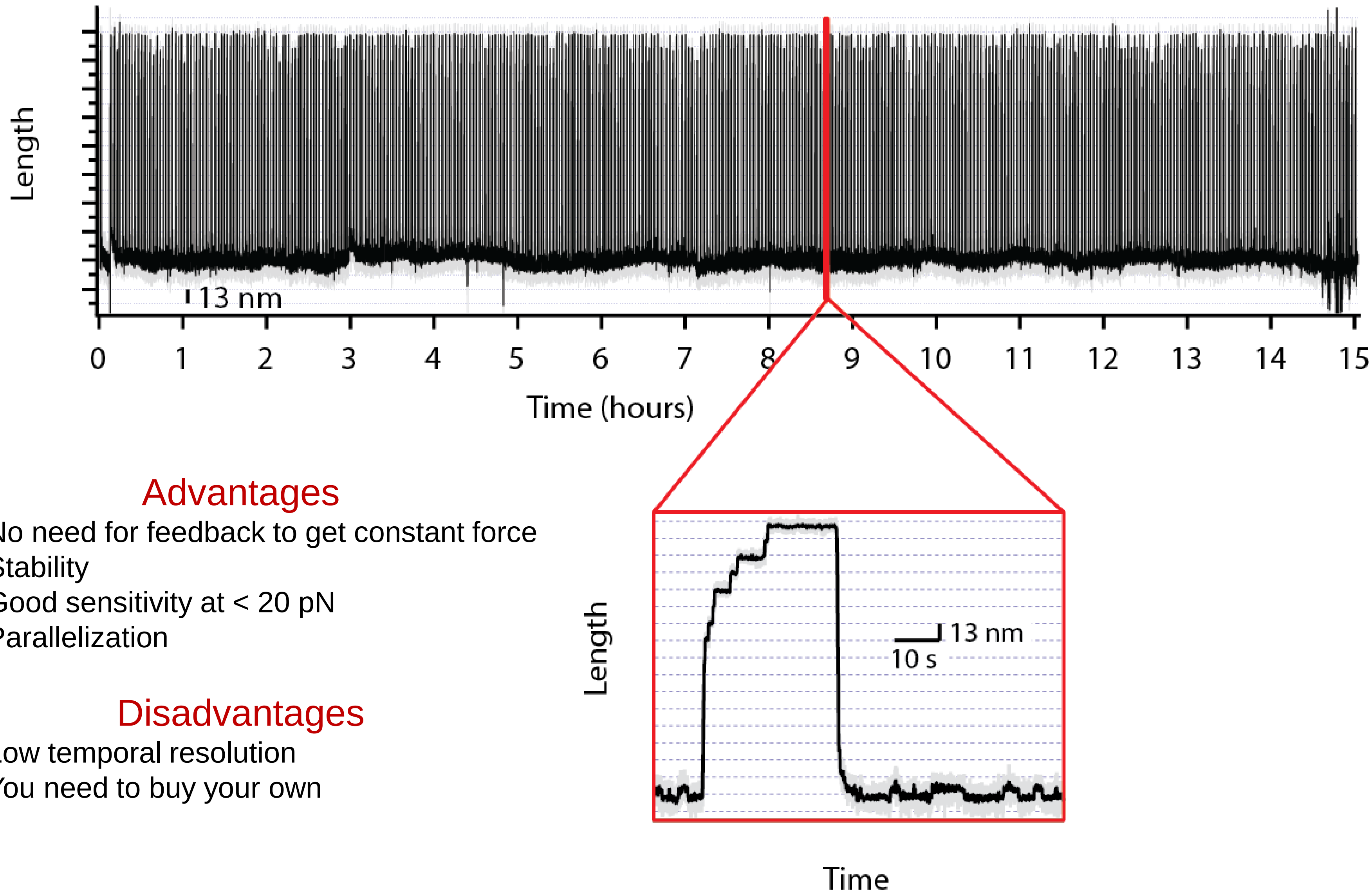


Magnetic tweezers





Magnetic tweezers to examine the mechanical properties of proteins



Advantages

- No need for feedback to get constant force
- Stability
- Good sensitivity at < 20 pN
- Parallelization

Disadvantages

- Low temporal resolution
- You need to buy your own

OT

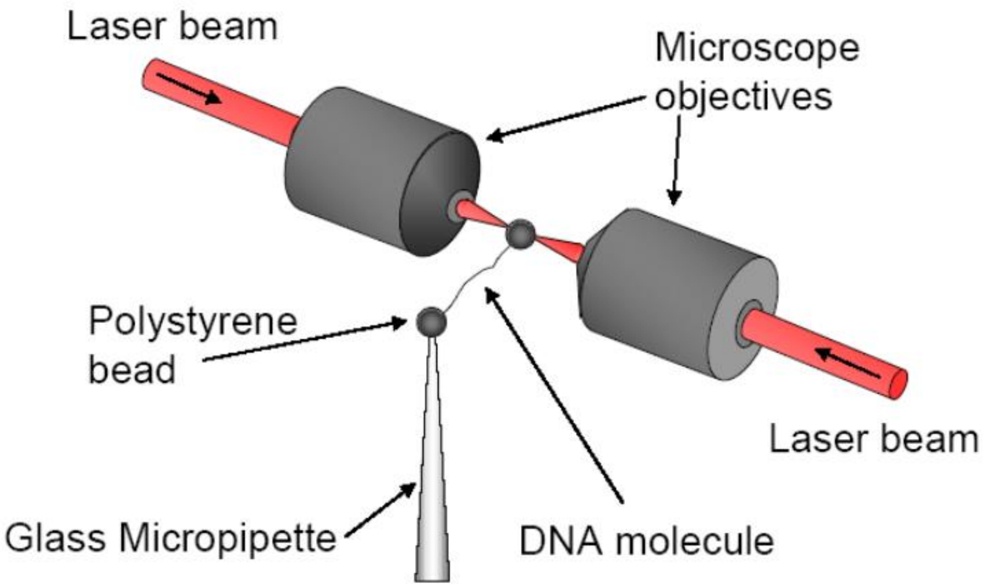
Optical tweezers

Folding-Unfolding Transitions in Single Titin Molecules Characterized with Laser Tweezers

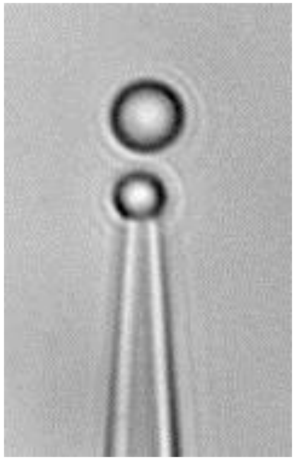
Miklós S. Z. Kellermayer,[*,†] Steven B. Smith,[*]
Henk L. Granzier,[‡] Carlos Bustamante[*]

Science (1997) 276, 1112

Trapping small objects using light



Simple trap



Optical tweezers

Double trap

https://youtu.be/gOA7wvycV-Q

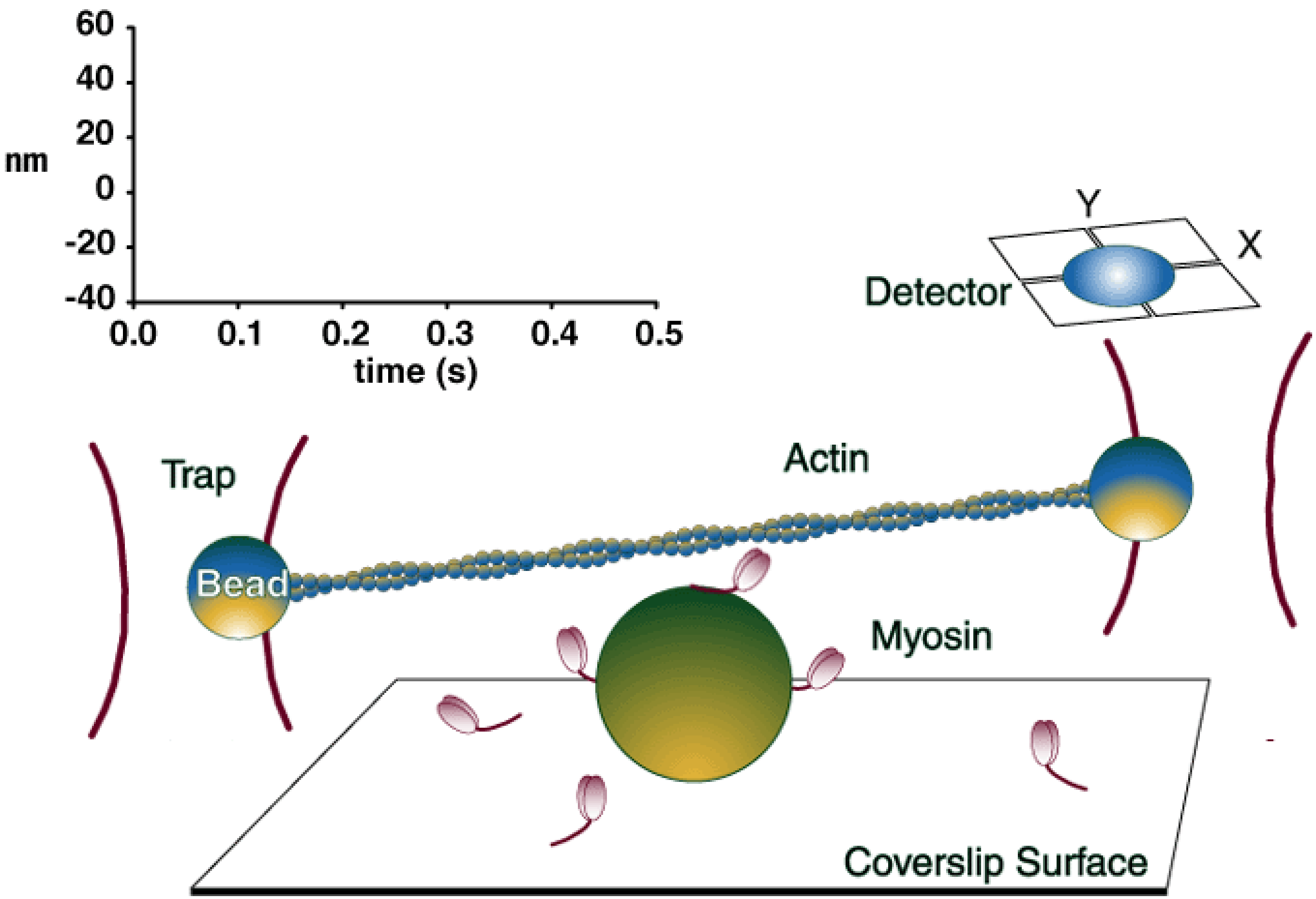
Advantages

- Good sensitivity at < 20 pN
- Controlled manipulation: versatility

Disadvantages

- Complex instrumentation for high resolution studies
- Need of molecular handles

Measuring the activity of molecular motors using OT



From single molecules to heart disease: take home messages

- Single-molecule methods provide new information that may be relevant to understand the pathophysiolgy of (heart) diseases.
- Many key biomolecules experience or produce mechanical force
- Single molecules behave stochastically
- Main single molecule manipulation techniques: AFM, MT, OT
- A new mindset and novel analysis tools

Recent findings in the field

RESEARCH ARTICLE

BIOCHEMISTRY

Contractility parameters of human β-cardiac myosin with the hypertrophic cardiomyopathy mutation R403Q show loss of motor function

10.1126/sciadv.1500511

Suman Nag,[1] Ruth F. Sommese,[1] Zoltan Ujfalusi,[2] Ariana Combs,[3] ~~Stephen Langer~~,[3] Shirley Sutton,[1] Leslie A. Leinwand,[3] Michael A. Geeves,[2] Kathleen M. Ruppel,[1,4]* James A. Spudich[1]*

Single Molecule Force Spectroscopy on Titin Implicates Immunoglobulin Domain Stability as a Cardiac Disease Mechanism*

Received for publication, July 16, 2012, and in revised form, December 10, 2012 Published, JBC Papers in Press, January 6, 2013, DOI 10.1074/jbc.M112.401372

Brian R. Anderson[‡§], Julius Bogomolovas[¶], Siegfried Labeit[¶], and Henk Granzier[§1]

More reading...

- Neuman, K. C. & Nagy, A. (2008) **Single-molecule force spectroscopy: optical tweezers, magnetic tweezers and atomic force microscopy**. *Nat Methods* **5**, 491-505.

- Popa, I., Kosuri, P., Alegre-Cebollada, J., Garcia-Manyes, S. & Fernandez, J. M. (2013) **Force dependency of biochemical reactions measured by single-molecule force-clamp spectroscopy**. *Nat Protoc* **8**, 1261-1276.

- Joo, C., Balci, H., Ishitsuka, Y., Buranachai, C. & Ha, T. (2008) **Advances in single-molecule fluorescence methods for molecular biology**. *Annu Rev Biochem* **77**, 51-76.

- Linke, W. A. & Hamdani, N (2014). **Gigantic business: titin properties and function through thick and thin**. *Circ Res* **114**, 1052-1068.

For any question or feedback:

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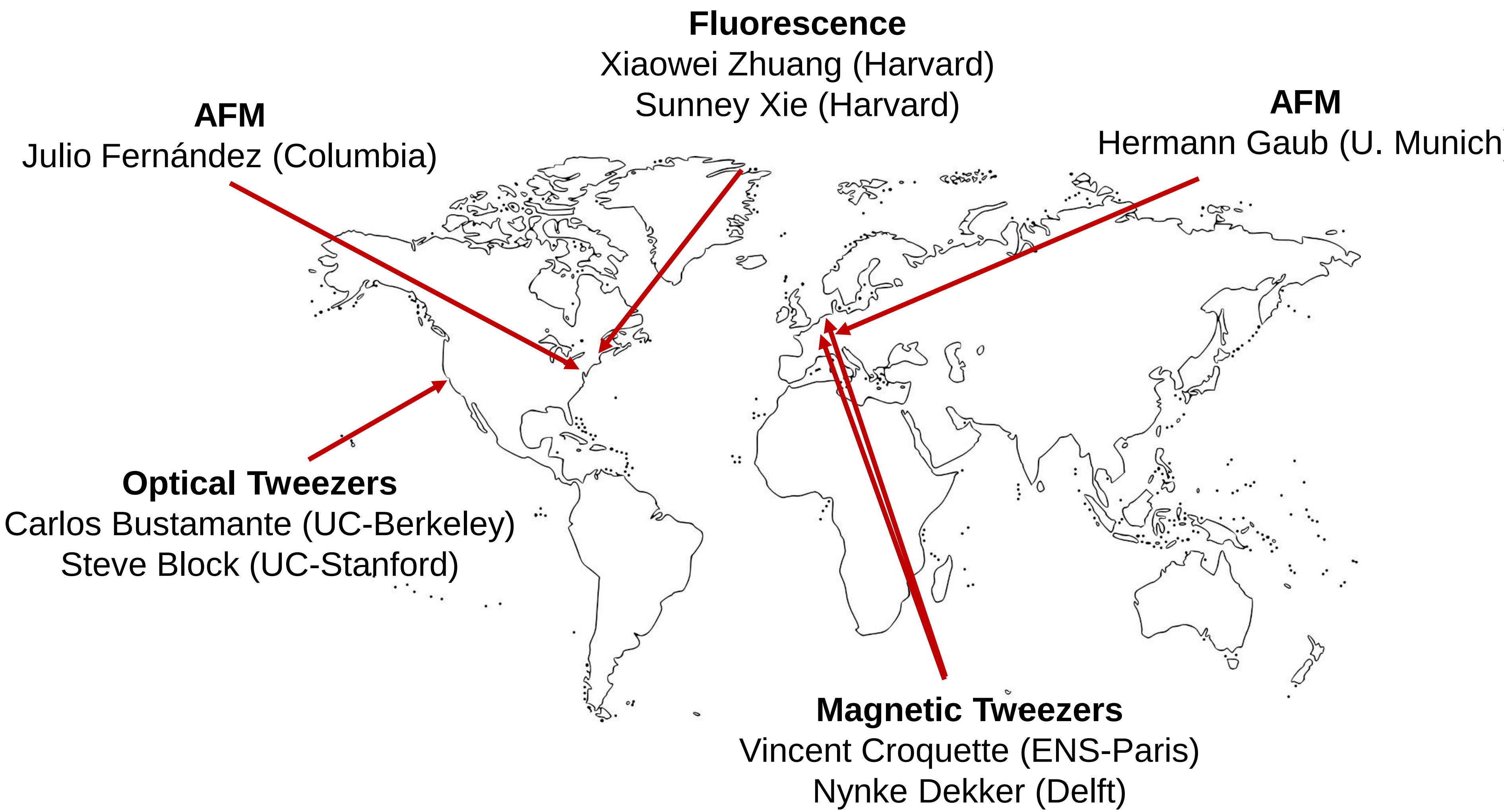
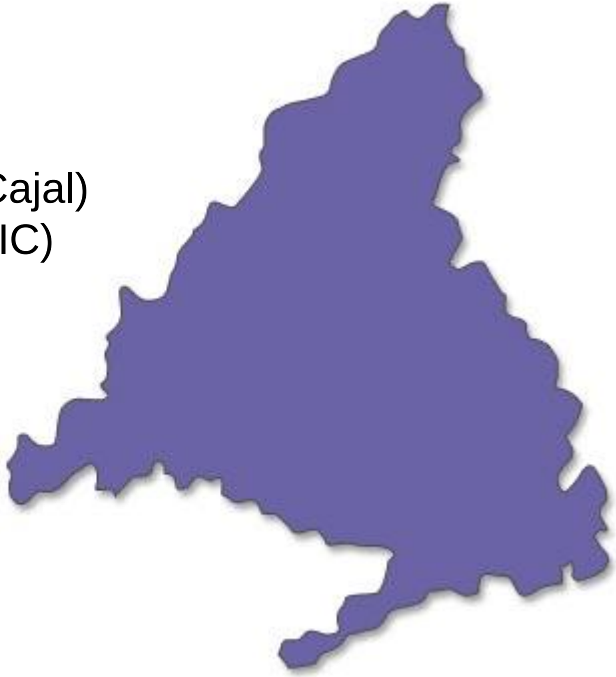
Some of the world-leading single-molecule laboratories



Single-molecule in Madrid

Optical Tweezers
J. Ricardo Arias-González (IMDEA-Nanociencia)
Borja Ibarra (IMDEA-Nanociencia)

AFM
Mariano Carrión-Vázquez (I. Cajal)
Jorge Alegre-Cebollada (CNIC)



Magnetic Tweezers
Fernando Moreno-Herrero (CNB)

Fluorescence

Also Félix Ritort (U. Barcelona, Optical Tweezers), Raúl Pérez-Jiménez (Nanogune, AFM)…

Mechanobiology Seminar Series at CNIC. If interested, send an e-mail to jalegre@cnic.es

From single molecules to heart disease

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Carlos III

Jorge Alegre-Cebollada